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Role of Tumor Necrosis Factor Alpha (TNF-a) In Hippocampal Neurodegeneration

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ROLE OF TUMOR NECROSIS FACTOR ALPHA (TNF-α) IN HIPPOCAMPAL NEURODEGENERATION

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This dissertation is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Prof. Abdu Adem

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Declaration of Original Work

I, Sara Shaaban Sharkawi Sayed, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Role of Tumor Necrosis Factor Alpha (TNF-α) in Hippocampal Neurodegeneration*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Prof. Abdu Adem, in the College of Medicine and Health Sciences at UAEU. This work has not been previously presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

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Abstract

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine with homeostatic and pathological roles in the central nervous system. The main objective of this dissertation is to study the impact of presence and absence of TNF-α on kainic acid (KA)-induced neurotoxicity at several time points (0.5 and 4 hr as well as 1, 3, 5, 15 and 30 days) to find out the possible mechanisms underlying its effects. KA (40 mg/kg) was given intranasally to TNF-α knockout (Ko) mice and C57BL/6 wild-type (Wt) mice. Seizure severity was scored and behavioral tests including Elevated Plus-Maze (EPM), open-field, and Y-maze were performed. The hippocampal levels of cytokines (IL-1β, IL-6, IL-12, IL-10), Insulin-like growth factor-I (IGF-I), and nerve growth factor (NGF) were assessed. Hippocampal oxidative stress markers including malondialdehyde, nitric oxide, glutathione (GSH), catalase and superoxide dismutase (SOD) were evaluated. Immunohistochemical methods were used to assess neurodegeneration and glial activation.

Compared with Wt-mice, TNF-α Ko mice were more susceptible to KA-induced neurotoxicity by showing rapid onset ($P<0.001$) and severe seizures ($P<0.01$). In EPM, TNF-α Ko mice showed changed risk assessment performance ($P<0.01$) especially at 30 days post KA. In open field test, TNF-α Ko mice showed significant hyperactivity at 3 and 30 days post KA treatment. In the Y-maze at 30 days post KA, TNF-α Ko mice showed significantly lower percent alternation compared to the respective KA-treated Wt-mice. Increased levels of proinflammatory cytokines (IL-1β, IL-6 and IL-12) as well as decreased levels of anti-inflammatory cytokines (IL-10) were observed in both strains following KA-

treatment. KA-treated TNF-α Ko mice showed more severe oxidative stress (*P<0.01*), lower IGF-I levels (*P<0.05*), and higher levels of β-NGF (*P<0.05*) compared to Wt-mice. Hippocampal GSH levels were significantly elevated in Wt-mice but not in TNF-α Ko mice, while, catalase and SOD activity were elevated (*P<0.001*) in TNF-α Ko mice. Hippocampal microglial activation and astrogliosis were significantly enhanced and persisted up to 30 days in TNF-α Ko mice compared with Wt-mice. Moreover, significant hippocampal CA3 neurodegeneration was observed 3 days post KA-treatment in both TNF-α Ko and Wt-mice compared to controls. The neurodegeneration was progressive and more significant (*P<0.01*) in TNF-α Ko mice compared with Wt-mice. Additionally, KA-treatment significantly up-regulated NFκB expression at 5 days post KA in TNF-α Ko mice. Taken together, our findings showed that deficiency of TNF-α worsens KA-induced neurotoxicity. These results highlighted the protective effects of TNF-α in KA-induced neurotoxicity and suggested that these neuroprotective effects may be through the regulation of microglial activation, induction of antioxidant defensive mechanisms and regulation of the NFκB signaling pathway.

Keywords: Tumor necrosis factor alpha (TNF-α), Neurodegeneration, Kainic acid, Neuroinflammation, Microglia, Growth factors, Oxidative stress, NFκB.

Title and Abstract (in Arabic)

دور تي إن إف ألفا (TNF-α) في موت الخلايا العصبية في الحصين

الملخص

يعتبر (TNF-α) من البروتينات والسيتوكينات المحفزة للالتهاب وله العديد من الوظائف التنظيمية بالإضافة إلى بعض التأثيرات المسببة للأمراض في الجهاز العصبي المركزي . ويعد الهدف الرئيسي من هذه الأطروحة هو دراسة تأثير وجود بروتين (TNF-α) على موت الخلايا العصبية المستحدث باستخدام حمض الكاينيك (Kainic acid) ودراسة تأثيراته على فترات زمنية مختلفة بهدف معرفة بعض الآليات المحتملة لتأثيراته.

ولتحقيق هذه الأهداف ، قمنا باستخدام نوعين من الفئران أحدهما يحمل الجين الخاص ببروتين (TNF-α) وينتجه ويسمى فئران النوع البري (Wt) والآخر لا يحمل الجين الخاص ببروتين (TNF-α) ومن ثم لا ينتجه ويسمى فئران (TNF-α Ko) . وقسمت الفئران إلى مجموعات تمثل الفترات الزمنية المختلفة للدراسة وأعطيت مادة حمض الكاينيك بجرعة (40 ملغم / كغم من وزن الجسم) عن طريق الأنف ، بينما تم إعطاء مجموعات التحكم ماءا مقطرا. وبعد الانتهاء من الجرعة كاملة قمنا بتسجيل شدة النوبات الصرعية (Seizures) التي تمر بها الفئران عقب التعرض لحمض الكاينيك . كما أجرينا بعض الاختبارات السلوكية للفئران (Elevated plus maze, Open Field and Y-maze) . إضافة لما سبق فقد قمنا باستخراج منطقة الحصين (Hippocampus) من أدمغة الفئران وقسنا فيها مستويات السيتوكينات المختلفة (IL-6, IL-12, IL-1β, IL-10) ، وكذلك مستويات عامل النمو المشابه للأنسولين (IGF-I) ، وعامل نمو الأعصاب (NGF) . كما تم قياس معدلات العديد من معاملات الإجهاد التأكسدي بما في ذلك مادة المالونداليديهيد (MDA) وأكسيد النيتريك و الجلوتاثيون (GSH) وإنزيم الكاتلاز (Catalase) وإنزيم (Superoxide dismutase SOD) في الحصين (Hippocampus) . واستخدمت الطرق المناعية لصباغة أنسجة المخ (Immunohistochemical methods) من أجل تقييم موت الخلايا العصبية ومدى نشاط الخلايا الغير عصبية في المخ (Neuroglia) .

ولقد أظهرت النتائج أن فئران (TNF-α Ko) أكثر عرضة وحساسية تجاه الإصابة العصبية الناجمة عن حمض الكاينيك مقارنة مع فئران النوع البري (Wt) . وظهر ذلك جليا في سرعة ظهور النوبات الصرعية (Seizures) وزيادة شدتها . وفي اختبارات السلوك ظهر تغير ملحوظ في قدرة فئران (TNF-α Ko) على تقدير الخطر خصوصا بعد ثلاثين يوما من التعرض لحمض الكاينيك بالإضافة إلى زيادة واضحة في النشاط الحركي لها مع تدهور ملحوظ في كفاءة الذاكرة القصيرة لهذه الفئران . وقد لوحظت زيادة مستويات السيتوكينات المحفزة للالتهاب (IL-6, IL-12, IL-1β) وكذلك انخفاض مستويات السيتوكينات المضادة للالتهاب (IL-10) في نوعي الفئران بعد التعرض لحمض الكاينيك . واتضح أن مستويات بروتين (IGF-I) كانت أقل بينما مستويات بروتين (NGF) كانت أعلى في فئران (TNF-α Ko) مقارنة بفئران النوع البري (Wt) بعد تعرضها لحمض الكاينيك .

وقد لاحظنا أيضا أن الإجهاد التأكسدي كان أكثر شدة في فئران (TNF-α Ko) . أما بالنسبة للدفاعات المختلفة للجسم في مواجهة الإجهاد التأكسدي ، فقد ارتفعت مستويات الجلوتاثيون (GSH) فقط في فئران النوع البري (Wt) وليس في فئران (TNF-α Ko) . وارتفع نشاط إنزيمي (Catalase & SOD) بشكل أوضح في فئران (TNF-α Ko) كآلية دفاعية ضد زيادة الإجهاد التأكسدي . كما تبين أن مستويات بروتين (NFκB) قد ارتفعت في فئران (TNF-α Ko) بعد 5 أيام من التعرض لحمض الكاينيك .

أما بالنسبة للخلايا غير العصبية (Microglia & Astrocytes) ، فقد لوحظ ازدياد نشاطها وفاعليتها خلال مدة الدراسة واستمرت مرحلة نشاطها لمدة شهر في فئران (TNF-α Ko) مقارنة بفئران النوع البري (Wt) . إضافة إلى ذلك فقد بدا موت الخلايا العصبية جليا في منطقة (CA3) من الحصين بعد ثلاثة أيام من التعرض لحمض الكاينيك في نوعي الفئران المستخدمة . ولوحظ أن موت الخلايا العصبية تدريجي ويزداد بمرور الوقت وهو أكثر شدة في فئران (TNF-α Ko) مقارنة بفئران النوع البري (Wt) .

وإجمالا ، فقد أظهرت هذه الدراسة أن نقص (TNF-α) يفاقم من موت الخلايا العصبية المستحدث باستخدام حمض الكاينيك . وتقدم نتائج هذه الدراسة إسهاما في إبراز التأثيرات الواقية التي يقلل بها بروتين (TNF-α) موت الخلايا العصبية الذي يسببه حمض الكاينيك . ولعل من الآليات المحتملة للتأثيرات الواقية

لبروتين (TNF-α) قدرته على تنظيم نشاط الخلايا غير العصبية (Microglia) و تحفيز الآليات الدفاعية ضد الإجهاد التأكسدي بالإضافة إلى ظبط وتنظيم مستويات بروتين (NFκB).

كلمات البحث المساعدة : بروتين (TNF-α) ، موت الخلايا العصبية ، حمض الكايينيك ، الالتهاب العصبي ، الخلايا غير العصبية (Microglia) ، عوامل النمو ، الإجهاد التأكسدي ، بروتين (NFκB) .

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Dedication

To my beloved mother, dear father, lovely husband and sweet kids

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List of Abbreviations

6-OHDA: 6-Hydroxyopamine

Aβ: β Amyloid peptide

AD: Alzheimer's Disease

AKT: Protein Kinase B

ALS: Amyotrophic Lateral Sclerosis

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA: One-way Analysis of Variance

ATP: Adenosine Triphosphate

BBB: Blood Brain Barrier

CA: Cornu Ammonis

CAT: Catalase

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

ELISA: Enzyme Linked Immunosorbent Assay

FJB: Fluoro-Jade B

GFAP: Glial Fibrillary Acidic Protein

GSH: Glutathione

HD: Huntington Disease

HRP: Horseradish Peroxidase

Iba-1: Ionized Calcium Binding Adaptor Molecule 1

IGF: Insulin-like Growth Factor

IL: Interleukin

KA: Kainic Acid

Ko: knockout

LPS: Lipopolysaccaride

MAPK: Mitogen Activated Protein Kinase

MDA: Malondialdehyde

MS: Multiple Sclerosis

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NADPH: Reduced form of Nicotinamide Adenine Dinucleotide Phosphate

NeuN: Neuronal Nuclear Antigen

NFκB: Nuclear Factor Kappa B

NGF: Nerve Growth Factor

NMDA: N-methyl-D-aspartate

NSAIDs: Non Steroidal Anti-Inflammatory Drugs

NO: Nitric Oxide

PBS: Phosphate Buffered Saline

PD: Parkinson's Disease

ROS: Reactive Oxygen Species

RNS: Reactive Nitrogen Species

SEM: Standard Error of the Mean

SOD: Superoxide Dismutase

TBARS: Thiobarbituric Acid Reactive Substances

TGF-β: Transforming Growth Factor Beta

TMT: Trimethyltin

TNF-α: Tumor Necrosis Factor Alpha

TNFR: TNF Receptor

Wt: Wild-type

Chapter 1: Introduction

1.1 Neurodegeneration

1.1.1 Definition

Neurodegeneration is the umbrella term for the progressive loss of structure or function of neurons, including death of neurons (Rubinsztein, 2006). A neurodegenerative disease is an irreversible deterioration in the intellectual and cognitive functions as a result of gradual and progressive loss of structure and/or function of neurons (Brown et al., 2005). Neurodegenerative diseases represent a wide group of central nervous system (CNS) diseases with different clinical and pathological features affecting specific neurons in the brain, mostly due to unknown causes, and progress in an unexpected manner. The most common neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) (Przedborski et al., 2003).

1.1.2 Factors Contributing to Neurodegeneration

In general, the major risk factor for developing neurodegenerative diseases is old age; however they can also affect young adults (Forman et al., 2004). As a result of the huge improvements in health care and medical research, the population lives longer, and an increasing number of elderly people will suffer from neurodegenerative diseases. The exact cause of neurodegenerative diseases is still debatable. Although the study of inherited familial cases has provided insight into the genetic factors (Shastry, 2003), many studies reported that an interaction between aging, environmental factors, and genetic predisposition contributes to the

development of neurodegenerative diseases (Campbell, 2004). Multiple components are linked to the pathogenesis of neurodegenerative diseases including protein aggregation (Rubinsztein, 2006), mitochondrial dysfunction and oxidative stress (Lin and Beal, 2006), diet components and neurotoxins (Brown et al., 2005), vascular disorders, excitotoxicity (Salinska et al., 2005), and inflammation (Wyss-Coray and Mucke, 2002).

1.1.3 Pathogenesis of Neurodegenerative Diseases

The pathogenesis and the mechanisms underlying neurodegeneration are still not completely defined. The shared characteristic of all neurodegenerative disorders is the selective and progressive loss of neurons in specific brain structures (Golde, 2009). Based on the study of patients with genetic forms of common and uncommon neurodegenerative diseases it can be inferred that the majority of these diseases feature a fairly long disease free period in which no pathological or clinical changes are noted. Subsequently, there is a phase that may last for years or even decades where the initiating pathology is detectable, but the patient remains symptom free. During this phase, additional pathological changes occur in the brain, including neuronal loss, atrophy, and synaptic dysfunction (Byrne et al., 2011). Thus, when symptoms become apparent there is typically extensive damage to either vulnerable brain regions in diseases that are characterized by more focal dysfunction or widespread damage to multiple brain regions (Byrne et al., 2011; Golde, 2009).

1.1.4 Neurodegeneration and Therapeutics

The majority of neurodegenerative diseases are difficult to diagnose accurately, and a clinical diagnosis is usually made through a process of elimination (Emard et al., 1995; Shaw et al., 2007). At present, the pharmacological therapy of neurodegenerative diseases is limited mostly to symptomatic treatments that do not alter the course of the underlying disease and have many side effects (Przedborski et al., 2003). Symptomatic treatment for PD, where the neurochemical defect produced by the disease is well defined, is relatively successful, and a number of effective agents are available. The available treatments for AD, HD, and ALS are much more limited in effectiveness (Shaw et al., 2007). In order to find effective treatments, we should understand the pathogenesis and the mechanisms underlying neurodegeneration, which are still not completely defined till now. An important goal of much current research in the pharmacology of neurodegenerative disorders is the identification of drugs that can slow the underlying degenerative process.

1.1.5 Effect of Neurodegeneration on Families and Society

Each year over 10 million people suffer from neurodegenerative diseases, with an expected 20% increase over the next decade. This increase in the aging population will exaggerate the level of emotional, physical, and financial burdens on patients, caregivers, and society (Atkinson, 2010; The Lancet, 2013).

1.1.6 Experimental Models of Neurodegeneration

Experimental animal models are necessary to understand the disease etiology, pathophysiology, compensatory mechanisms and progression, to establish new

therapeutic strategies, and to extend the pharmacological approach towards a more effective treatment for patients with neurodegenerative diseases.

Several experimental models have been established to study neurodegeneration. In vitro models involved the use of neonatal neuron cell cultures or adult brain slice cultures (Legradi et al., 2011). In vivo models involved surgical brain lesions, administration of neurotoxins (MPTP, Kainic acid, Domoic acid), or genetic manipulation (Butler et al., 2003; Kirik and Bjorklund, 2003; Waerzeggers et al., 2010). Most of these models result in neurodegeneration in targeted regions of the brain that will mimic what happened in various neurodegenerative disorders.

Unfortunately, no single animal model is entirely useful in reproducing the complete spectrum of pathological changes observed after neurodegeneration and further research is necessary to fully reveal the acute and chronic changes that occur after neurodegeneration. Although it is difficult to create a comprehensive animal model for neurodegenerative diseases, the current models are still useful to give us different parts from the whole picture, and hopefully when these parts are put together we could know more about the reality of the neurodegeneration.

1.2 Neurodegeneration and Inflammation

Inflammation in the brain is termed neuroinflammation. It is a fundamental response generated to protect the CNS; however, uncontrolled or prolonged neuroinflammation is potentially harmful and can result in cellular damage. This is particularly relevant to neurodegenerative diseases, which are typified by evidence of microglial activation and neuroinflammation (Doty et al., 2015; Frank-Cannon et al.,

2009). Previously, inflammation was viewed as only a passive response to neuronal damage. However, increasing reports demonstrate that inflammation is capable of actively causing neuronal death and damage (Streit et al., 2004; Sundaram and Gowtham, 2012). Thus, while the triggers of various neurodegenerative diseases are diverse, inflammation may be a basic mechanism driving the progressive nature of multiple neurodegenerative diseases. Several cell types have been listed as contributors to inflammation-mediated neurodegeneration, but microglia are considered the critical component (Block and Hong, 2005). Since neurodegeneration involves a chronic inflammatory process. the inflammatory molecules may be crucially implicated at various stages in the pathological cascade of neurodegenerative diseases (Glass et al., 2010). Thus, anti-inflammatory treatment may slow down the process of neurodegeneration. Consequently, it is important to focus on the role of inflammatory mediators in the pathology of neurodegeneration.

1.2.1 Sources of Inflammatory Mediators in the CNS

The brain is considered as an immune privileged organ, due to its compartmentalization and separation from the peripheral blood system by the blood brain barrier (BBB). The impermeability of the BBB prevents the entrance of immunoglobulins and the invasion of leukocytes from blood. However, many immunocompetent molecules, such as various cytokines and chemokines and their receptors, have been identified within the CNS (Nakamura, 2002). These findings confirm that there are resident cells in the CNS that work as immune cells and produce these molecules (Nakamura, 2002; Prinz et al., 2014). Most neurodegenerative diseases are characterized by both local inflammation from resident cell types in the brain and by the infiltration of leucocytes from the

periphery (Kurkowska-Jastrzebska et al., 1999). While infiltrating peripheral immune cells can be significantly toxic to neurons (Wu and Proia, 2004), leukocyte infiltration is not always associated with neurotoxicity (Trifilo and Lane, 2003). This indicates a critical role for the local glial cells (astroglia and microglia) in the inflammatory response associated with neurodegeneration.

1.2.2 Glial cells

In the CNS, there are three kinds of glial cell other than neurons: astrocytes, oligodendrocytes, and microglia. These cells are surrounded by capillary endothelial cells and ependymal cells. The endothelial cells form the BBB that isolates central cells from peripheral tissues by maintaining highly stable conditions within the CNS (Nakamura, 2002).

1.2.2.1 Astroglia

In the normal brain, astroglia play essential roles in providing glia-neuron contact, maintaining ionic homeostasis, buffering excess neurotransmitters, secreting neurotrophic factors, and serving as a critical component of the blood–brain barrier (Aloisi, 1999; Fiacco et al., 2009). Although the pro-inflammatory function of astroglia is not as prominent as that of microglia (Streit et al., 1999), astroglia become activated in response to immunologic challenges or brain injuries (Colangelo et al., 2014). Astroglia also produce a host of trophic factors (Lindsay, 1994), which are crucial for the survival of neurons. However, activated astroglia become hypertrophic, exhibit increased production of glial fibrillary acidic protein (GFAP), and form glial scars, which prevent axonal regeneration (Fiacco et al., 2009).

1.2.2.2 Microglia

In all tissues of the body, there is a population of resident macrophages. These cells form a first line of defense against tissue injury or infection, and rapidly respond to alterations in tissue homeostasis. Microglia are the major resident immune cells in the CNS parenchyma, where they constantly survey the microenvironment and produce factors that influence surrounding astrocytes and neurons (Glass et al., 2010). They were originally described by del Rio-Hortega (1932) as a unique cell type differing in morphology from other glia and neurons, comprising approximately 12% of the brain. They are responsible for the innate immune response in the brain. They perform general maintenance and clean cellular debris (Beyer et al., 2000). Additionally, microglia have active roles in late embryonic brain development and early postnatal brain maturation, where microglia enforce the programmed elimination of neural cells (Barron, 1995; Prinz et al., 2014).

1.2.3 Dynamics of Microglial Cells

Microglial cells have been classically described to exist in two states, resting and activated (Morgan et al., 2004). However, this binary definition has been revised to make way for more complex ideas. Microglia, in the healthy CNS, are not truly "resting", but engaged in environmental surveillance, constantly sampling areas around them to maintain homeostasis (Nimmerjahn et al., 2005). Once microglia encounter changes in homeostasis, infection or injury, they enter an "activated" state in order to regulate CNS innate immunity and initiate appropriate responses, such as inflammation (Perry et al., 1995). It is now recognized that activated microglia can exist broadly in two different states (Colton, 2009). The first is classical activation,

which is typified by the production of inflammatory cytokines and reactive oxygen species, while the second is a state of alternative activation, in which microglia take on an anti-inflammatory phenotype involved in wound repair and debris clearance (Cherry et al., 2014; Gordon, 2003).

1.2.3.1 M1 and M2 Microglia

Once microglial cells are activated, they up-regulate a variety of surface immune receptors, such as toll-like receptors, the major histocompatibility complex, and complement receptors (Ransohoff and Brown, 2012). They also undergo dramatic morphological changes from resting ramified cells to activated amoeboid microglia (Kreutzberg, 1996). Besides morphological changes and surface molecule upregulation, activated microglial cells secrete a host of soluble factors. Some of these factors, such as the glia-derived neurotrophic factor, are potentially beneficial to the survival of neurons (Aloisi, 1999). The majority of factors produced by activated microglia, however, are proinflammatory. These include the cytokines TNF-α, IL-6, IL-1β, interferon-γ (IFN-γ) and several chemokines (Boche et al., 2013; Lee et al., 1993), free radicals such as nitric oxide (NO) (Liu et al., 2002), and superoxide (Colton and Gilbert, 1987), fatty acid metabolites such as eicosanoids, and quinolinic acid (Liu and Hong, 2003). The cytokine production is essential for the polarization of microglia into a classically activated, “M1”, state (Mills et al., 2000). IFN-γ produced from Th1 cells was found to be instrumental in polarizing macrophages to M1 (Nathan et al., 1983). Microglia and astrocytes have also been observed to produce IFN-γ (Kawanokuchi et al., 2006), demonstrating that microglia can control their own polarization through autocrine and paracrine means. Normally, it is supposed that the classical activation of microglia is protective, self-limiting and

is under strict control; resolving once infection has been eradicated or the tissue damage has been repaired (Glass et al., 2010). However, unregulated, long-term, or chronic inflammation can lead to tissue destruction (Perry and Teeling, 2013).

Alternatively activated macrophages, "M2 microglia", express cytokines and receptors that are implicated in inhibiting inflammation and restoring homeostasis. This includes production of IL-10 to downregulate inflammatory cells, extracellular matrix protecting proteins like YM1, polyamines for wound repair, neuroprotective factors like IGF-I or brain derived neurotrophic factor and higher levels of receptors associated with phagocytosis, such as scavenger receptors (Martinez et al., 2009). Just as the Th1 cytokine IFN-γ has been associated with induction of proinflammatory M1 macrophages, the Th2 cytokine IL-4 has been associated with M2, or alternative activation (Stein et al., 1992). Interestingly, it appears that when there is a lack of M2 cell differentiation in the CNS, problems can arise because of lower levels of neuroprotective factors like IGF-I or brain derived neurotrophic factor, which microglia produce (Amor et al., 2010). Indeed, many investigators are starting to recognize the importance of M1/M2 dynamics in diseases characterized by chronic neuroinflammation.

1.2.3.2 Activated Microglia May Be Protective or Toxic

A lot of controversy exists regarding whether microglia are neuroprotective or neurotoxic when activated. In addition to producing cytotoxic factors such as superoxide, nitric oxide, and TNF-α in response to immunological stimuli, microglia are also reported to increase neuronal survival through the release of trophic and anti-inflammatory factors. Thus, it is likely that microglia can serve both functions,

depending on several factors ranging from the progression of the disease state to the type of stimulus. Although some inflammatory stimuli induce beneficial effects (e.g., phagocytosis of debris and apoptotic cells), and inflammation is linked to tissue repair processes, over-activation of microglia and consequent uncontrolled inflammation may result in production of neurotoxic factors that amplify underlying disease states (Block and Hong, 2005; Glass et al., 2010).

1.2.4 Microglial Activation in CNS Diseases

In the past, it was a general understanding that microglia are consequently activated after neuronal damage to phagocytose the damaged neurons, after which the postneuronal space is occupied by the proliferation of reactive astrocytes (gliosis or astrocyte scarring). However, Morioka and coworkers observed in (1991) that the microglial activation began before the neuronal degeneration in an animal model of transient brain ischemia. Since then, the possibility has been examined in various experimental systems that "microglial activation" may be a cause of neuronal degeneration rather than a consequence of it.

In vitro, microglial cells represent the large majority of proliferating cells in slice cultures after exposure to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or to N-methyl-D-aspartate (NMDA) (Dehghani et al., 2004). An increase in microglia reactivity and proliferation close to degenerating neurons was also observed in the brain of kainate-treated rats (Tooyama et al., 2002) and in models of cerebral ischemia (Dempsey et al., 2003). These cells were localized preferentially close to the damaged and dying neurons.

Studies using cell culture and animal models have demonstrated that excessive quantities of individual factors produced by activated microglia can be deleterious to neurons (Chao et al., 1992; McGuire et al., 2001). Furthermore, individual factors often work together to induce neurodegeneration. Xie and coworkers in (2002) showed that peroxynitrite, a product of superoxide and NO, is a major mediator of neurotoxicity induced by lipopolysaccharide (LPS) or β-amyloid peptide (Aβ 1-42).

The involvement of microglial activation in the pathogenesis of several neurodegenerative diseases was initially postulated based on the postmortem analysis of the brains of patients with AD and PD. Reactive microglia were found to colocalize with neuritic plaques in the cortical region of AD brains (Rogers et al., 1988). In PD brains, large numbers of reactive microglia were found in the substantia nigra (McGeer et al., 1988). In addition to AD and PD, results from both in vivo and in vitro studies have established an association of microglial activation with the pathogenesis of ALS and MS (Dickson et al., 1993; Raine, 1994).

The growing evidence that microglia may be a key player in the process of neurodegeneration encouraged researchers to target microglia and inflammation as a therapeutic intervention for neurodegenerative diseases. For example, Administration of aminopyridazines is shown to both attenuate glial inflammation and result in reduction of neuronal neurotoxicity in the murine intraventricular human Aβ 1-42 infusion model (Craft et al., 2004). In a separate study, non-steroidal anti-inflammatory drug (NSAID) treatment in mice over-expressing Aβ was able to lower

Aβ deposition, inhibit microglial activation, and provide neuroprotection (Yan et al., 2003).

There has been some success with clinical studies investigating the effects of anti-inflammatory therapy against cognitive decline in AD patients (Perry et al., 2003). Many epidemiologic studies have indicated that the use of anti-inflammatory drugs reduces the incidence and slows the progress of Alzheimer's disease (McGeer and McGeer, 1999). A prospective population-based cohort study of about 7000 individuals revealed that the relative risk of AD decreases to 0.2 with the use of NSAIDs for more than 2 years (in t' Veld et al., 2001). It is possible that the target of such anti-inflammatory drugs is microglia.

Evidence that taking NSAIDs may slow the progression or delay the onset of AD indicates, at a minimum, that inflammation and microglia are critical for the ongoing process of neurodegeneration in AD. A major unresolved question is whether inhibition of these responses will be a safe and effective means of reversing or slowing the course of disease.

1.2.5 Mechanisms of Microglial Activation

Microglia can be activated by two mechanisms: (1) direct stimulation of microglia from environmental or endogenous toxins; (2) activation through a reactive microgliosis process. In general, dying or damaged neurons have the potential to activate microglia, regardless of how the neurons were damaged. Reactive microgliosis (Figure 1) is the process in which damaged neurons activate microglia to initiate a self propelling cycle of neuron-death. There are diverse triggers that are

able to activate microglia directly and stimulate their proliferation to exert their neurotoxicity. These triggers (Figure 2) include immunological insult, such as LPS; environmental toxins and endogenous disease proteins (Block and Hong, 2005).

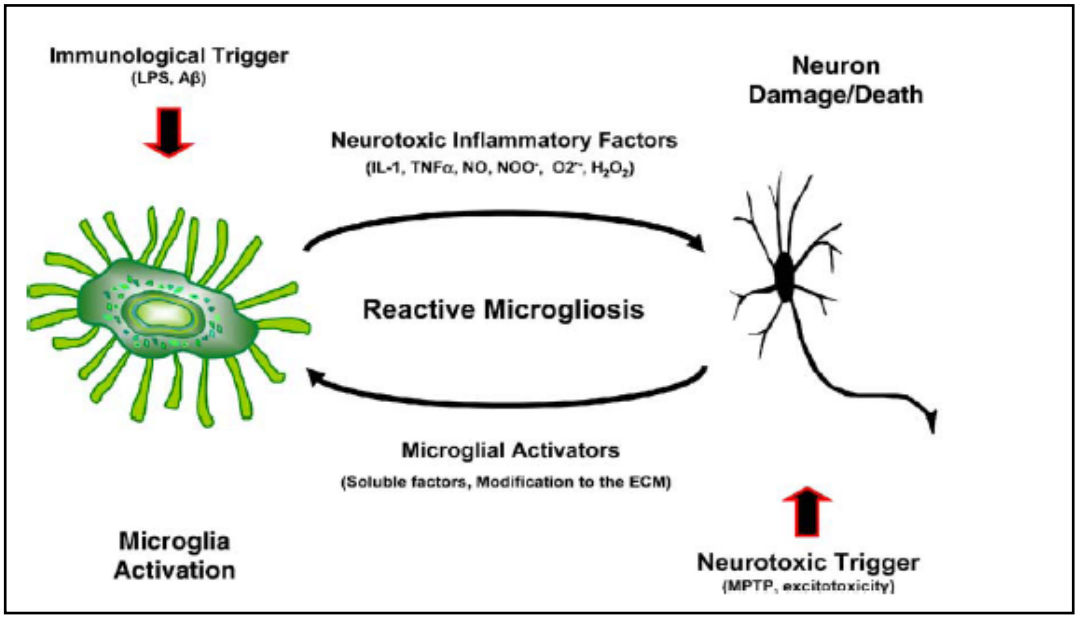


Figure 1: Reactive microgliosis (Block and Hong, 2005)

LPS is a widely used as a powerful tool for the activation of microglia and peripheral immune cells. Although LPS has no known direct toxic effect on neurons, it activates microglia to release a host of neurotoxic factors to induce neuronal death (Liu et al., 2002). Neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), cause direct damage to dopaminergic neurons and causes reactive gliosis. Activation of microglia in turn exacerbates the neurodegenerative process (Wu et al., 2002). Some agents that are known to be associated with various neurodegenerative diseases exhibit a "mixed mode" mechanism of neurotoxicity, Aβ and α-synuclein protein. It appears that the overall neurotoxic effect of these mixed mode toxins includes both direct neurotoxicity and indirect toxicity through the activation of microglia (McMillian et al., 1994).

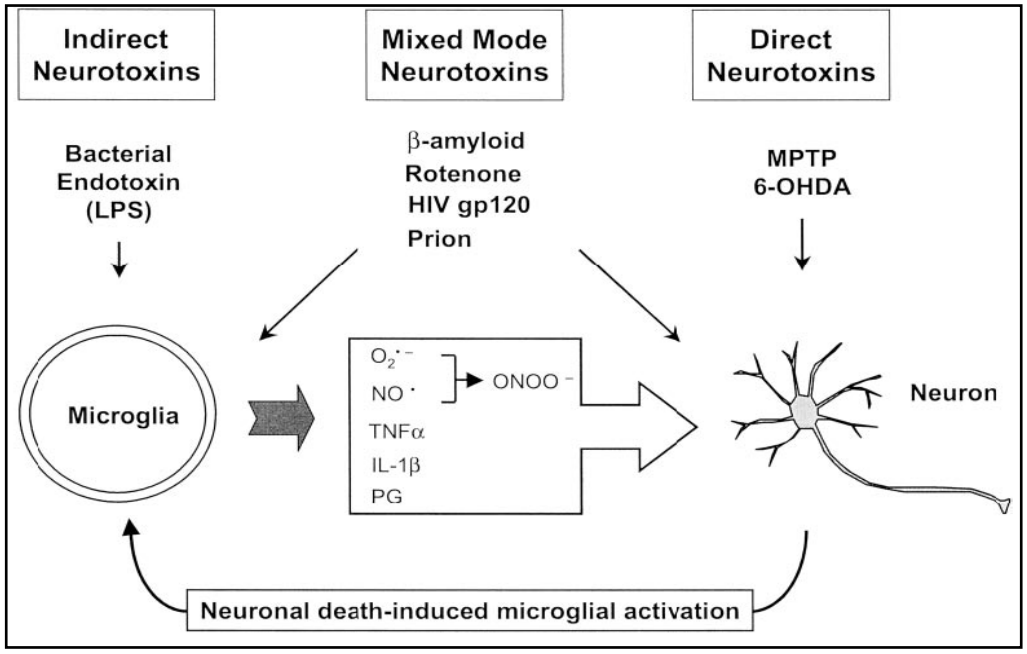


Figure 2: Types of neurotoxins (Liu and Hong, 2003)

The intracellular signaling mechanism of microglial activation and inactivation was not fully studied. It is possible that the unique collection of membrane channels of microglia ,including the presence of an inward rectifying K^+ channel and the absence of an outward rectifying K^+ channel is one route by which microglia may be sensitive to their ionic microenvironment (Kettenmann et al., 1990). Furthermore, adenosine triphosphate (ATP) is rapidly released from cells during acute degeneration and several studies suggest that a purinergic receptor binding ATP may also rapidly activate microglia (Perry et al., 1995). It has been shown that activated microglia express the transcription factor nuclear factor kappa B (NF-κB) during immune-mediated activation (Kaltschmidt et al., 1994).

1.2.6 Common Characteristics of M1 Microglial Activation

1.2.6.1 Release of Neurotoxic Factors

Across several toxins, it is clear that the first event is the production of reactive oxygen species (ROS), which includes the extracellular superoxide anion (Gao et al., 2002). The increase in the production of ROS is rapid, usually occurring within minutes, and is typically measured in microglia 10–30 min after LPS addition. The microglial ROS response is followed by the release of cytokines (such as TNF-α and IL-1β), nitric oxide, and prostaglandin E2 that peaks around 6–12 h (Gao et al., 2002). Experiments using different mutant mice deficient in NADPH oxidase, iNOS, COX-2, or TNF-α receptors all resulted in reduced neurotoxicity (Wang et al., 2004), indicating that the individual proinflammatory factors released are sufficient, but not mandatory, for neurotoxicity.

1.2.6.2 NADPH Oxidase

NADPH oxidase is the predominant source of microglial extracellular ROS production in response to multiple and diverse stimuli (Wu et al., 2005). NADPH oxidase is a membrane-bound enzyme that catalyzes the production of superoxide from oxygen. This enzyme is dormant in resting phagocytes but is activated when the cell is activated (Babior, 2000).

Oxidative stress is a common characteristic shared across numerous neurodegenerative diseases (Perluigi et al., 2005), suggesting a basic and similar mechanism underlying diverse neurodegenerative pathology. Further, NADPH oxidase is upregulated in neurodegenerative diseases such as PD (Wu et al., 2003).

1.2.6.3 ROS and Proinflammatory Factors

Intracellular ROS are critical for the activation of microglia and the enhancement of the production of proinflammatory factors. There is increasing support that intracellular ROS can also function as second messengers to regulate several downstream signaling molecules, including protein kinase C, mitogen activated protein kinase (MAPK) and NF-κB (Konishi et al., 1997). Min and coworkers in (2004) demonstrated that the production of IL-1β, TNF-α and iNOS are attenuated by the addition of the NADPH oxidase inhibitor, diphenyleneiodonium. Furthermore, NADPH oxidase inhibitors and catalase are shown to suppress LPS-induced expression of cytokines (IL-1β, IL-6, and TNF-α), iNOS expression, MAP kinases, and NF-κB phosphorylation (Pawate et al., 2004).

Accumulating evidence supports that activation of NADPH oxidase contributes to microglia-mediated neurotoxicity through two mechanisms: (1) Production of extracellular ROS that is toxic to neurons. (2) Activation of Increasing microglial intracellular ROS, which enhances the production of proinflammatory factors that are toxic to neurons (Block and Hong, 2005).

1.3 Cytokines

Cytokines are a family of low molecular weight, multifunctional polypeptides acting as humoral regulators that modulate the functional activities of individual cells and tissues under normal, pathological and toxicological conditions. They are primarily involved in host responses to disease or infection (Dinarello, 2000). They act as mediators of inflammation and regulation of cell growth and differentiation.

Originally called lymphokines and monokines to indicate their cellular sources, but it became clear that the term “cytokine” is the best description, since nearly most nucleated cells are capable of synthesizing these proteins and, in turn, of responding to them (Dinarello, 2000). They include interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), chemokines and growth factors. Many cytokines show synergistic or overlapping activities, and in some cases share related receptors (Rothwell and Strijbos, 1995).

1.3.1 Proinflammatory and Anti-inflammatory Cytokines

Some cytokines clearly promote inflammation and are called proinflammatory cytokines (IL-1β, TNF-α, IL-6, and chemokines), whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines (IL-3, IL-4, IL-10 and TGF-β) (Kolesnick and Golde, 1994; Smith et al., 2012; Viviani et al., 2004). A “balance” between the effects of proinflammatory and anti-inflammatory cytokines is thought to determine the outcome of disease, whether in the short term or long term. Some studies suggested that susceptibility to a disease is genetically determined by the balance or expression of proinflammatory or anti-inflammatory cytokines (Dinarello, 2000).

1.3.2 Expression and Action of Cytokines in the Brain

Cytokines have been involved in the modulation of several neurological functions and dysfunctions. The numerous cytokines affecting the CNS have two possible origins: (1) they originate from the peripheral immune organs and cross the BBB, (2) they are produced by glial cells (astrocytes and microglia) and certain

neurons. For many cytokines, glial cells seem to be the primary producers in the CNS (Zhang and Zhu, 2011). The expression of most cytokines is tightly regulated and these factors are usually produced only after cell activation in response to an induction signal (Viviani et al., 2004). They are hardly detectable in the CNS under physiological conditions, but both pro- and anti-inflammatory cytokines become rapidly upregulated by pathological events, like Alzheimer's disease, excitotoxicity, exposure to neurotoxins, LPS injection or viral infection (Gabellec et al., 1995; Marquette et al., 1996; Minami et al., 1991; Smith et al., 2012; Sriram et al., 2002).

1.4 Tumor Necrosis Factor Alpha (TNF-α)

TNF-α is a multi-functional potent proinflammatory cytokine that can regulate many cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism. Its actions range from an important role in lymphoid organ development and maturation of the humoral immune response (Pasparakis et al., 1996), to its better known involvement in the pathogenesis of infectious, inflammatory and autoimmune diseases (Guerder et al., 1994; Vanden Berghe et al., 2014). TNF-α was originally discovered in the mouse serum during endotoxemia and recognized for its anti-tumor activity (Carswell et al., 1975). It is synthesized as a 26-kDa transmembrane monomer (mTNF-α) that undergoes proteolytic cleavage by the TNF-α converting enzyme to yield a 17-kDa soluble TNF-α molecule (s TNF-α). The proteolysis is mediated by TNF-α converting enzyme (Black et al., 1997). Both sTNF-α and mTNF-α can affect biological and metabolic responses, suggesting that mTNF-α may mediate paracrine and autocrine signals, leaving sTNF-α to mediate endocrine effects (Grell et al., 1999).

Its role in the CNS was not observed until 1987, when microglia was found to produce TNF-α (Frei et al., 1987). At present it is well established that apart from the microglial cells, TNF-α can also be synthesized and released in the brain by astrocytes and some populations of neurons (Probert, 2015; Viviani et al., 2004).

1.4.1 TNF-α Receptors

The pleiotropic actions of TNF-α are mediated through two distinct cell surface receptors: TNFR1 (p55) and TNFR2 (p75) (Wajant et al., 2003). They are membrane glycoprotein receptors that specifically bind TNF-α, but the two receptors differ in their expression profiles, ligand affinity, cytoplasmic tail structure, and downstream signaling pathway activation. Signaling of TNF-α requires that TNFRs preassemble on the cell membrane as trimers prior to ligand binding. This trimerization occurs through the intracellular cytoplasmic tail of the receptors (Chan et al., 2000). TNF-α signaling through TNFR1 and TNFR2 can elicit a variety of cellular responses depending on many factors including the metabolic state of the cell and the adaptor proteins present in the cell. These differences result in a number of responses including inflammation, proliferation, cell migration, apoptosis, and necrosis (Eissner et al., 2004).

1.4.1.1 Cellular distribution of TNFRs

TNFR1 is expressed in most cell types, and can be activated by binding of either sTNF-α or mTNF-α, with a preference for sTNF-α; whereas TNFR2 is expressed primarily by cells of the immune system and by endothelial cells, and is preferentially activated by mTNF-α (Grell et al., 1998). In the brain, both TNFRs are

expressed by neurons and glia (Dopp et al., 1997). The differential patterns of localization of TNFRs in neuronal and glial cells, their state of activation and the down-stream effectors, all are thought to play an important role in determining whether TNF-α will exert a beneficial or harmful effect on CNS (Figiel, 2008).

1.4.1.2 TNFR1-Mediated Signaling

Only TNFR1 contains a cytoplasmic death domain (DD) and may directly induce apoptosis. Binding of the TNF trimer to the extracellular domain of TNFR1 permits the assembly of the TNFR1 signaling complex through the dissociation of silencer of death domains (SODD) and subsequent binding the adaptor protein TNF receptor-associated death domain (TRADD) (Ware et al., 1996). TRADD recruits other adaptor proteins: receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2) and FAS-associated death domain (FADD) (Hsu et al., 1996).The latter two proteins recruit key enzymes that are responsible for initiating TNFR1 signaling events. For instance, caspase-8 is recruited by FADD leading to the formation of the death-inducing signaling complex (DISC), and triggering activation of the executioner caspases through the extrinsic apoptosis pathway (Schneider-Brachert et al., 2004). Caspase-8 also triggers the intrinsic apoptosis pathway by cleaving the proapoptotic Bcl-2 family members Bax and Bid to initiate mitochondrial-induced apoptosis (Wang et al., 2006). On the other hand TRAF2 recruits cellular inhibitor of apoptosis protein-1 and 2 (cIAP-1 and cIAP-2), two anti-apoptosis proteins that also have ubiquitin protein ligase activity. Moreover, TRAF2 may activate MAPK pathway leading to the activation of c-Jun N-terminal kinase (JNK) that phosphorylates c-Jun, increasing its transcriptional activity. Finally, the protein kinase RIP is critical to the activation of the transcription factor NF-κB

(Walczak, 2011). Therefore TNF-α binding to TNFR1 may result in activation of apoptosis, proliferation, or transcriptional activity (Figiel, 2008).

1.4.1.3 TNFR2-Mediated Signaling

Signaling through TNFR2 activates inflammatory and prosurvival signaling pathways through recruitment of TRAF1 and TRAF2 adaptor proteins and subsequent activation of cIAPs and the NF-κB pathway (Rao et al., 1995; Walczak, 2011). TNFR2 has also been shown to activate phosphatidylinositol 3-kinase dependent signaling to promote neuron survival (Marchetti et al., 2004). TNFR2 does not contain a DD, and thus, unlike signaling through TNFR1, TNFR2 activation does not directly lead to caspase activation. However, studies with TNF-α receptor-specific neutralizing antibodies showed that stimulation of TNFR2 relies on the induction of endogenous, membrane-bound TNF-α, which subsequently activates TNFR1 (Grell et al., 1999). TNFR2 can promote TNFR1 signaling by enhancing the association between TNF-α and TNFR1 via a ligand passing mechanism (Tartaglia et al., 1993). Figure 3 summarizes the TNF-α signaling through both TNFR1 and TNFR2.

1.4.2 TNF-α in the CNS

In the CNS, TNF-α is considered the principal mediator of neuroinflammation. TNF-α signaling have several important functions within the CNS, including injury-mediated microglial and astrocyte activation, and regulation of BBB permeability, febrile responses, glutamatergic transmission, and synaptic

plasticity (Boulanger, 2009; Merrill, 1991; Santello et al., 2011; Stellwagen and Malenka, 2006).

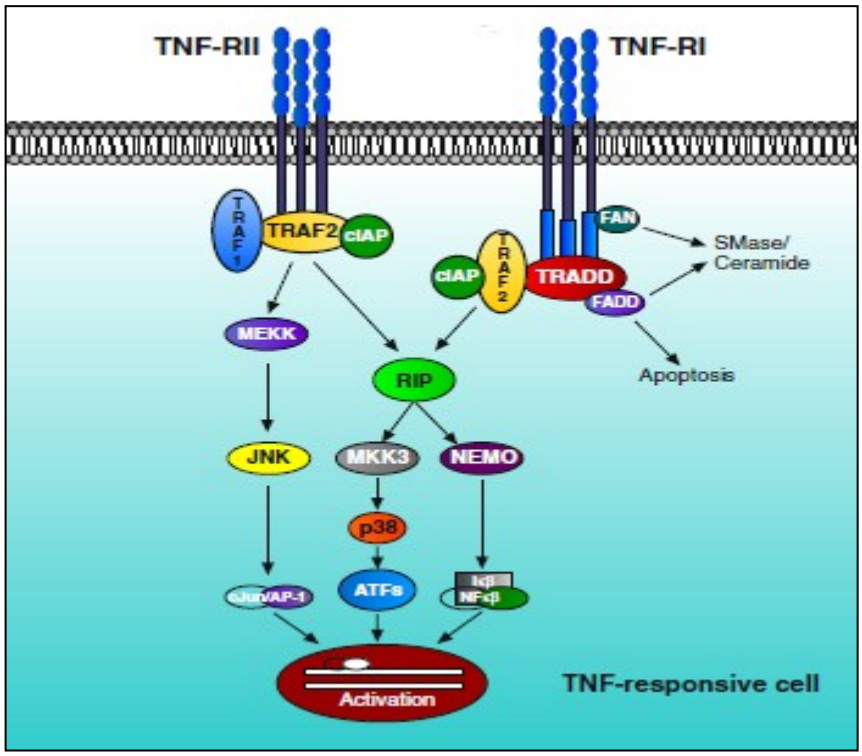


Figure 3: TNF-α signaling through TNFR1 and TNFR2 (Montgomery and Bowers, 2012)

1.4.3 TNF-α in Brain Disorders

1.4.3.1 Neurotoxic Effects of TNF-α

TNF-α can potentiate excitotoxicity by two mechanisms. In combination with sub-threshold glutamate levels, TNF-α can potentiate glutamate excitotoxicity directly through activation of glutamate-NMDA receptors (Zou and Crews, 2005) and localization of AMPA receptors to synapses (Leonoudakis et al., 2004), and indirectly by inhibiting glial glutamate transporters on astrocytes (Santello et al., 2011).

Elevated levels of TNF-α have been associated with the pathological effects of a variety of infectious, neurological, neurodegenerative, and neurotoxic conditions (Probert, 2015), including ischemia (Feuerstein et al., 1994), bacterial meningitis (Leist et al., 1988), cerebral malaria (Grau et al., 1989), traumatic brain injury (Goodman et al., 1990), multiple sclerosis (Raine et al., 1998), Alzheimer's disease (Alvarez et al., 2007) and Parkinson's disease (Mogi et al., 2000).

TNF-α immunoreactivity has been reported on neurons, astrocytes, microglia, and endothelial cells, and is present within perivascular spaces (Botchkina et al., 1997). Administration of exogenous TNF-α markedly exacerbates ischemic injury in vivo, and acute inhibition of TNF-α action, by administration of TNF-α soluble receptor or a neutralizing antibody to TNF-α markedly attenuates ischemic brain damage in rat and mouse (Barone et al., 1997). A number of preclinical and clinical studies in various disease models and in chronic neurodegenerative conditions suggest that targeting TNF-α action in the brain may be an attractive disease-modifying strategy to slow progression or attenuate severity of the disease (McCoy and Tansey, 2008).

1.4.3.2 Neuroprotective Effects of TNF-α

While several lines of evidence suggest a neurotoxic role for TNF-α in the CNS, it does not appear to be strictly neurotoxic. In addition to its key role in maintaining CNS homeostasis, TNF-α is known to influence survival, differentiation, proliferation, and growth. These features highlight a potential protective role for this cytokine (Sriram and O'Callaghan, 2007). It is important to know that TNF-α do not cause neuronal death in healthy brain tissue or normal neurons even at very high

doses (Venters et al., 1999). This failure to induce death directly may be because TNF-α is not neurotoxic per se.

Several studies showed that TNF-α can protect against brain injury. It has been shown to promote reparative remyelination in an experimental model of demyelination (Plant et al., 2005). In this case, TNF-α appears to promote the survival of oligodendrocytes, thereby, facilitating remyelination. Preconditioning with TNF-α protected neurons against ischemic injury (Ginis et al., 2002; Lambertsen et al., 2009), suggesting that TNF-α is involved in the development of ischemic tolerance. TNF-α also mediates neuroprotection in response to acute nitric oxide excitotoxicity (Turrin and Rivest, 2006).

Low doses of TNF-α had a neuroprotective effect on AMPA receptor-mediated excitotoxicity (Bernardino et al., 2005). In this study, the neuroprotection was found to be mediated by TNFR2, whereas the exacerbating action on AMPA toxicity was mediated by TNFR1. TNF-α also showed a protective role against Aβ peptide toxicity in rat hippocampal cultures (Barger et al., 1995) when administered before the toxic insults. More findings suggested that TNFR2 signaling protects against excitotoxicity. Specifically, cortical neurons from mice that overexpress TNF-α in the CNS were protected from glutamate toxicity as were wild type and TNFR1-deficient mice pretreated with TNF-α or agonistic TNFR2 antibodies (Marchetti et al., 2004). Consistent with those findings, neurons derived from TNFR2-deficient mice were susceptible to both TNF-α and glutamate-induced death. (Marchetti et al., 2004).

These observations strongly suggest that one potential approach to lessen adverse effects of anti-TNF therapies in the CNS may be to selectively target TNFR1 signaling with localized delivery of inhibitors which spare TNFR2-mediated signaling. As sTNF-α signals preferentially through TNFR1, selective inhibition of sTNF-α signaling may be advantageous. This approach may allow efficient neutralization of sTNF-α in the desired region without eliciting collateral damage to TNFR2/ mTNF-α-dependent processes in regions of the brain where mTNF-α has important homeostatic functions. A second strategy is to increase TNFR2 expression levels through gene therapy to allow protection of neuronal populations that are highly sensitive to sTNF-α/TNFR1- mediated toxicity (McCoy and Tansey, 2008).

Some studies showed that TNFR1 and TNFR2 may have opposite effect on neurons, with the former having damaging effect and the latter being neuroprotective (Fontaine et al., 2002). Studies in mice lacking only one type of TNFRs revealed that TNFR1 signaling pathway is more important than TNFR2 one in mediating neuroprotective actions of TNF-α after acute brain insults (Lu et al., 2008). This study showed that mice lacking TNFR1 exhibit more severe seizure activity, hippocampal neurodegeneration and increased microglial activation, suggesting that TNF-α exerts its protective role through TNFR1 signaling (Lu et al., 2008). In addition, TNFR2 involvement in chemical-induced apoptosis of murine hippocampal dentate granule neurons has been reported (Harry et al., 2008). In the latter study, they employed trimethyltin (TMT), a well known neurotoxicant, to induce neuronal injury accompanied by microglial activation and increased production of TNF-α.

In (1996), Bruce and colleagues reported that mice deficient in both receptors displayed greater neuronal damage following either ischemic or kainic acid induced excitotoxic damage. They found that damage was accompanied by increased oxidative stress as well as reduced level of manganese superoxide dismutase (Mn-SOD), so they suggested that TNF-α may play a beneficial role by stimulating antioxidant pathway. On the other hand, mice lacking both TNFRs were protected against the dopaminergic neurotoxicity of MPTP (Sriram et al., 2002). These paradoxical findings (Figure 4) suggested that TNF-α was capable of mediating differential effects in specific brain areas. While deficiency of TNF receptors blocked MPTP neurotoxicity in the striatum, the lack of these receptors rendered the hippocampus vulnerable to injury (Sriram et al., 2006).

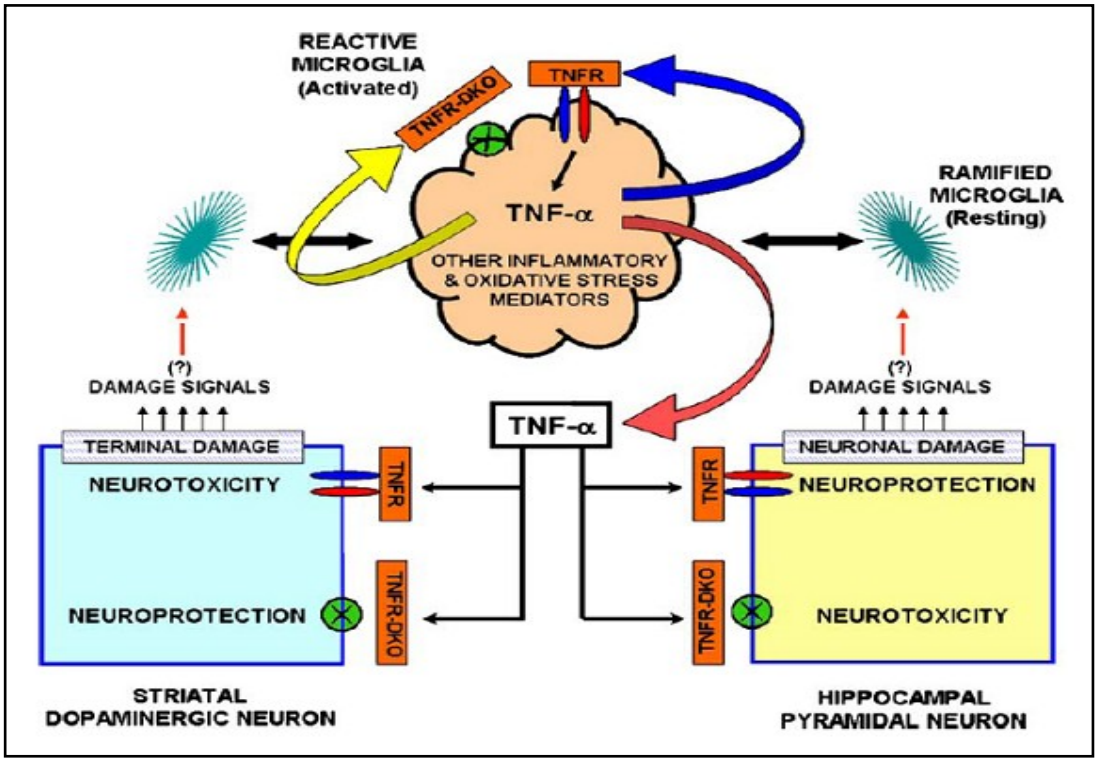


Figure 4: Paradoxical effects of TNF-α in different brain regions (Sriram and O'Callaghan, 2007)

1.5 Kainic Acid Induced Neurodegeneration Model

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. It is associated with cognition, memory, and movement (Cacabelos et al., 1999) and it is involved in early development of the human CNS (Bardoul et al., 1998). Over stimulation of glutamate receptors causes neuronal injury or death by a mechanism termed excitotoxicity. Excitotoxicity is thought to play a key role in the induction of neuronal cell death occurring after brain trauma and in neurological disorders, including neurodegenerative diseases (Meldrum, 2000). Activation of glutamate receptors causes an influx of sodium and calcium ions. The high intracellular levels of calcium then initiate signaling cascades within susceptible neurons that cause neuronal death (Choi, 1988).

Excitotoxic cell death is commonly induced experimentally by the administration of kainic acid (KA), a potent agonist of the AMPA/kainate class of ionotropic glutamate receptors. Systemic administration of KA in rodents results in recurrent seizures and subsequent degeneration of selective populations of neurons in the hippocampus, specifically CA3 and CA1 regions (Sperk et al., 1983). KA administration was shown to be associated with increased production of reactive oxygen species (ROS), mitochondrial dysfunction, activation of microglia, and disruption of calcium signaling (Benkovic et al., 2006).

These studies suggest that KA-induced excitotoxicity can be used as a model for elucidating mechanisms underlying inflammation and oxidative stress in neurodegenerative diseases (Wang et al., 2005; Zhang and Zhu, 2011). However, Schauwecker and Steward in (1997) showed that certain commonly used strains of

mice, specifically C57BL/6 and BALB/c, are resistant to KA-induced neuronal death. Subcutaneous doses of KA less than 40 mg/kg do not result in neuronal damage in the hippocampus of C57BL/6 mice. Doses higher than 40 mg/ kg, cause mild histopathological changes in the mouse hippocampus, but only at the expense of the survival rate, which is less than 25% (Schauwecker and Steward, 1997). As C57BL/6 mice are used for most transgenic studies, then resistance to KA-induced neuronal death makes it difficult to study KA-induced neurodegeneration in transgenic and knockout mice. Thus, a model of intranasal administration of KA was adopted and was found to be effective in these mice strains, specifically C57BL/6 mice (Chen et al., 2002; Zheng et al., 2011).

1.6 Research Problem and Rationale for the Study

In brief, TNF-α is a proinflammatory cytokine that exerts both homeostatic and pathophysiological roles in the CNS. TNF-α acts as a key player in the initiation and orchestration of inflammation in the periphery as well as in the CNS (Probert, 2015). Several reports suggested a neurotoxic role for TNF-α. High levels of TNF-α have been observed in several CNS injuries such as ischemia, trauma, infection, neurodegeneration, and chemically induced neurotoxicity (Alvarez et al., 2007; Feuerstein et al., 1994; Goodman et al., 1990; Leist et al., 1988; Mogi et al., 2000). TNF-α also was found to potentiate excitotoxic injury (Barone et al., 1997). Whether TNF-α signaling actively contributes to or limits neuronal injury in these disorders was not established. Most of these reports concluded that targeting TNF-α signaling would be a good therapeutic target to modify the progress of neurodegeneration (McCoy and Tansey, 2008). On the other hand, several studies suggest neuroprotective properties for TNF-α. It was found that absence of TNF-α worsens

CNS infections (Figiel, 2008). In addition, knockout mice lacking both TNF receptors or TNFR1 receptors only showed exacerbated ischemic and excitotoxic brain damage (Bruce et al., 1996; Fontaine et al., 2002; Lu et al., 2008). Furthermore, dysregulated TNF-α signaling has been implicated in the initiation and/or progression of a number of human diseases (Figiel, 2008; Probert, 2015). These studies provided an important factor to be considered for therapeutic approaches involving complete neutralization of TNF-α for treatment of various disorders, such as rheumatoid arthritis, because these treatments my result in increased susceptibility to neuronal injury. Hence, a controversy exists regarding the role of TNF-α in neurodegeneration.

We have to consider that these studies involved different models of neurodegeneration targeting different areas of the brain and to appreciate different cellular composition (neuron/microglia) and receptor expression (TNFR1/TNFR2). Some of these studies are in vitro studies lacking the interaction between neurons and surrounding glial cells. In addition, most of these studies showed different degrees of TNF-α signaling inhibition with very few studies using TNF-α knockout mice.

In KA-induced neurotoxicity model, most of studies were performed at one or two time points (3 days, 5 days or maximum 7 days) (Zhang et al., 2007; Zhang et al., 2012; Zhang et al., 2008). Thus, they observed the effects of TNF-α at theses time points but at the same time they may miss other effects in very early or late time points.

In this thesis, we wanted to be specific, focusing on the role of TNF-α in hippocampal neurodegeneration. We applied intranasal kainic acid induced neurodegeneration model for the first time in TNF-α Ko mice. We also covered a wide range of time points (0.5 and 4 hr as well as 1, 3, 5, 15 and 30 days) to catch very early changes as well as delayed ones in the course of the hippocampal neurodegeneration process.

1.7 Hypothesis

The hypothesis of this study is that TNF-α exerts protection for hippocampal neurons, hence removal of TNF-α will worsen hippocampal neurodegeneration.

1.8 Aims of the Study

The main objective of this study is to clarify the neuroprotective role of TNF-α in kainic acid induced hippocampal neurodegeneration.

Specific aims:

1. To establish a model of neurodegeneration in male TNF-α knockout mice and C57BL/6 mice using intranasal administration of KA.
2. To follow up the neurodegeneration process at different time points (0.5 and 4 hr as well as 1, 3, 5, 15 and 30 days) post KA treatment.
3. To investigate the impact of the presence and absence of TNF-α on learning, memory and behavior of mice in KA-induced neurodegeneration.
4. To investigate the impact of the presence and absence of TNF-α on neuronal cell death and microglial activation in mice with KA-induced neurodegeneration.
5. To investigate the impact of the presence and absence of TNF-α on oxidative stress, cytokines and growth factors in mice with KA-induced neurodegeneration.
6. To find a possible mechanism(s) for the role of TNF-α in KA-induced neurodegeneration.

Chapter 2: Materials and Methods

This study was approved by the Research Ethics Committee (Institutional Review Board) at the College of Medicine and Health Sciences, UAE University.

2.1 Animals

Adult age-matched male TNF-α knockout (Ko) (n = 170) and C57BL/6 wild type (Wt) mice (n = 130), (6 to 8 weeks old) are used in the present study. The TNF-α Ko mice had been generated through targeted disruption of the TNF-α gene and being backcrossed to the C57BL/6 strain (Marino et al., 1997). It was reported that females as well as aged C57BL/6 mice were more sensitive to KA-induced neurodegeneration (Zhang et al., 2008). In order to avoid any confounding factors we preferred to use male adult mice in this study. All mice were housed on a 12 hr light-dark schedule with water and food available ad libitum. The KA induced excitotoxic model in mice was approved by the CMHS Research Animal Ethics Committee, UAEU. All efforts were made to minimize the number of animals used and their sufferings.

2.2 KA Administration and Seizure Activity Assessment

Male TNF-α Ko and C57BL/6 Wt mice (n = 50) were partially anesthetized with Isoflurane and held on their backs by hand. KA dissolved in distilled water, (10 mg/1.3 ml) was slowly and gently dropped by micropipette into the nostrils of the mice at a dose of 40 mg/kg body weight (Chen et al., 2002; Lu et al., 2008). A total of 40 µl of KA solution was delivered over 10 min (20 µl for each naris, with a break of 2 min between instillation into each nostril). For volumes of more than 40 µl of

KA, the first 40 μl of KA was administered over 10 min, with a break for 10 min. The next 40 μl of KA was then administered. Age- and body weight-matched control TNF-α Ko and C57BL/6 Wt mice received the same amount of vehicle (Distilled Water) intranasally. Mice were observed by two different examiners continuously for 5 hours to record the onset and extent of seizure activity. Seizures were rated according to the criteria described by Ben-Ari (1985) with modifications: 0, normal; 1, immobilization; 2, rearing and falling; 3, seizure for less than 1 hr; 4, seizure for 1-3 hr; 5, seizure for more than 3 hr; and 6, death.

Different groups of mice from both strains are kept for different time points; 0.5 hr and 4 hr as well as 1, 3, 5, 15 and 30 days after KA treatment, to investigate the time course and the role of TNF-α in hippocampal neurodegeneration.

2.3 Behavioral Assessments

TNF-α Ko mice and Wt mice (n = 15-22) are exposed to three behavioral tests before and after KA treatment according to the experimental design shown in (Figure 5). All behavioral tests were carried out between the hours from 9:00 to 15:00 in a dimly lit room, into which the animals were brought in their home cage 1 hr prior to the start of the test.

-3 day	**EPM before KA**
-2 days	**Open Field before KA**
-1 day	**Y-Maze before KA**
0 day	**KA treatment**
.....	.....
.....	.....
3 days before sample collection	**EPM after KA**
2 days before sample collection	**Open Field after KA**
1 day before sample collection	**Y-Maze after KA**
Endpoint	**Sacrificing mice**

Figure 5: Experimental Design for Behavioral Tests

2.3.1 Elevated Plus Maze Test (EPM)

This test gives a measure of anxiety level and it is done 3 days before KA treatment and 3 days before sacrificing the mice of both strains. It is based on the assumption that normally mice prefer a closed environment to an open space (Pellow et al., 1985). The EPM apparatus consists of two open (30 × 5 cm) and two closed arms of the same size Plexiglas, with 15 cm high walls. The arms were constructed of white acrylic radiating from a central platform (5 × 5 cm) to form a plus sign. The entire apparatus was elevated 40 cm above the floor. The floor of the arms was smooth. The test was initiated by placing each mouse on the central platform facing one of the open arms. An arm entry was defined as the entry of all four paws into the arm. The number of entries into the open and closed arms and the time spent on the open and closed arms were recorded over a 5-min test period. The maze apparatus was cleaned with water then 70% ethanol and dried after each trial.

2.3.2 Open Field Test

Open-field activity was measured two days before KA treatment and two days before sacrificing the mice of both strains. The open field test was used to measure exploratory behavior and spontaneous motor activity of the animals (Denenberg, 1969). The apparatus consisted of a transparent acrylic plastic (Plexiglas) box (35x35x17 cm) in which the floor was subdivided into 64 equal squares (4.38x4.38 cm). The box was placed under white light, and a mouse was gently placed in the center of the box. Locomotor activity and rearing were recorded every 3 min for a period of 9 min. Locomotor activity was evaluated by counting the number of times the mouse crossed the floor squares with both hind paws. Rearing was evaluated by counting the number of times the mouse was vertical with the forepaws raised. The enclosure was washed with water and then 70% ethanol between each test.

2.3.3 Y-Maze Test

Spontaneous alternation performance was tested using a symmetrical Y-maze. The Y-maze was used for identification of discrimination learning, spatial alteration tasks, and working memory of rodents (Hefco et al., 2003). This test is done one day before KA treatment and one day before sacrificing the mice of both groups. Each mouse was placed at the end of one arm of the Y-maze and was allowed to explore freely through the maze during a 6-min session. The sequence and total number of arms entered were recorded. Arm entry was considered to be complete when the hind paws of the mouse had been completely placed in the arm. The spontaneous alternation score (percentage) was calculated as the number of

alternations (i.e., consecutive entries into all three arms) divided by the total possible of alternations (i.e., total number of arms − 2) and multiplied by 100.

2.4 Histopathological Analysis

At the end of different time points experiments, all mice (n = 7-9) were anesthetized with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were extracted, fixed in 4% paraformaldehyde and kept in 10% sucrose until being frozen. Coronal sections showing the hippocampus area from -1.15, - 1.94 and - 2.80 mm, respectively, relative to the bregma were prepared according to the information in Franklin's brain atlas. At least 3 sections in the hippocampus area from each animal were stained by Fluoro-Jade B (FJB) to evaluate degenerating neurons.

2.5 Immunohistochemistry of Brain Sections

Fixed frozen hippocampal sections were prepared as described for histopathological analysis. At least 3 sections in the hippocampus area from each animal (n = 7-9) were used for each antibody staining. After washes with Tris buffer, the sections were blocked by "protein block" at room temperature for 30 min. Subsequently, they were exposed to rabbit antibodies to ionized calcium binding adaptor molecule-1 (Iba-1), rabbit antibodies to glial fibrillary acidic protein (GFAP) and rabbit antibodies against neuronal nuclear antigen (NeuN), followed by staining with either fluorescent (For GFAP) or the avidin-biotin technique (Iba-1 and NeuN). Omission of primary antibodies served as negative control.

2.6 Hippocampal Tissue Homogenate

At the end of different time points, all mice (n = 9-14) were anesthetized with sodium pentobarbital. Brains were extracted; hippocampi are dissected and kept immediately in liquid nitrogen, then stored in −70 °C until being analyzed. Hippocampal samples were homogenized (1:10 W/V ratio) in ice-cold Thermo protein extraction reagent® (T-PER) buffer, supplanted with 1:100 protease inhibitor cocktail. After sonication for 5×3 s, the samples were incubated for 30 minutes on ice to facilitate cross-linking, then centrifuged at 15,000 rpm (eppendorf® 5417R) for 30 min at 4 °C and the supernatant was collected and stored at −70 °C until use.

2.7 Cytokine Analysis

Protein concentrations were determined by using the protein assay kit and the levels of cytokine (TNF-α, IL-6, IL-12, Il-1β and IL-10) were assessed using ELISA (Mosmann and Fong, 1989). Briefly, standard 96-well ELISA plates (NUNC, Roskilde, Denmark) were incubated with the captured antibody, which binds the cytokine of interest, at 4 °C overnight. The next day the plates were blocked by incubation for 2 hr at RT with 1% bovine serum albumin in PBS. Serial dilutions of known amounts of standard protein ranging from 0 to 2,000 pg/ml were applied in duplicate for the standard curve. Wells containing the standard proteins and supernatants of hippocampal tissue homogenates were incubated at 4 °C overnight as specified by the protocol. They were then incubated with the corresponding biotinylated detecting antibody for 1 hr at RT. An avidinylated antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant was incubated for 30 min at RT. Tetramethylbenzidine at 100 µl per well was used to develop color. The reaction

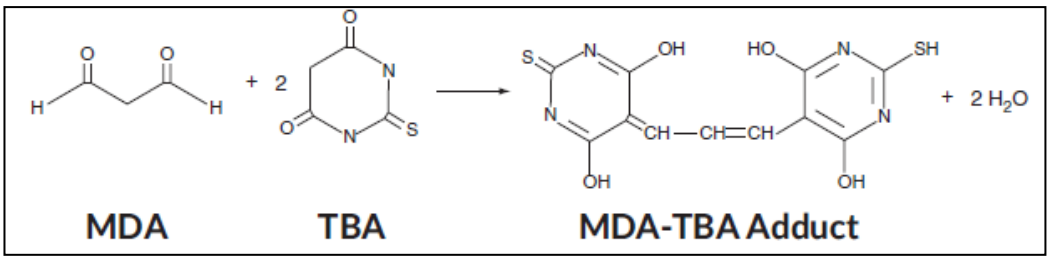
was terminated with 2N sulfuric acid and the absorbance was recorded at 450 nm in a plate reader within 10 min after stopping of the reaction.

2.8 Oxidative Stress Analysis

2.8.1 Malondialdehyde (MDA)

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxidation is used as an indicator of oxidative stress in cells and tissues. The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation (Yagi, 1998).

Cayman's TBARS Assay Kit was used to assay lipid peroxidation in hippocampal tissue homogenates. An MDA-TBA adduct (Equation 1) was formed by the reaction of MDA and Thiobarbituric Acid (TBA) when placed in boiling water of 90-100°C and acidic conditions. The adduct was then measured colorimetrically at 540 nm wavelength using the plate reader Infinite M200pro (Tecan®).

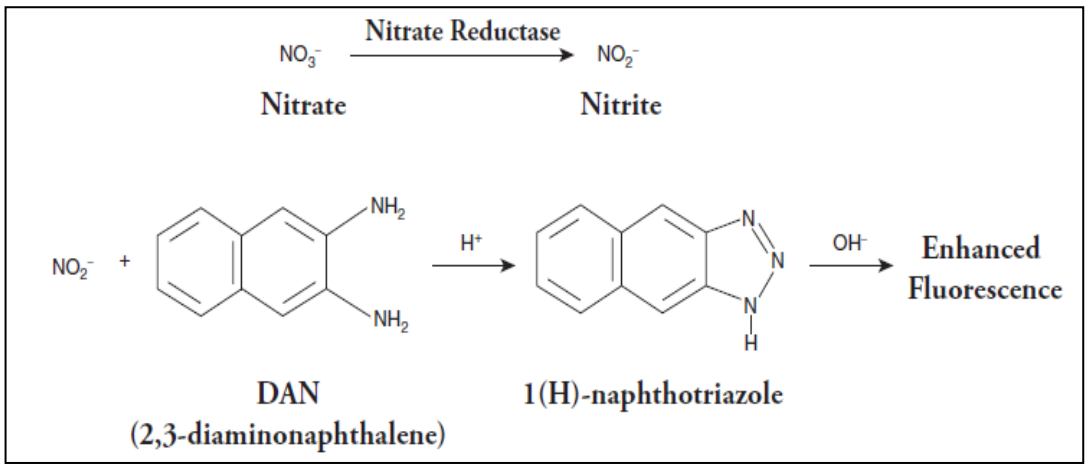


Equation 1: MDA-TBA adduct

2.8.2 Nitric Oxide Production

Nitric oxide (NO) is produced in trace quantities by neurons, endothelial cells, platelets and neutrophils in response to homeostatic stimuli. NO is also produced by other cells (macrophages, fibroblasts, hepatocytes) in micromolar concentrations in response to inflammatory or mitogenic stimuli. The final products of NO in vivo are nitrite (NO_2^-) and nitrate (NO_3^-). The best index of total NO production is the sum of both NO_2^- and NO_3^-.

Cayman's Nitrate/Nitrite Fluorometric Assay kit provides measures total nitrate/nitrite concentration in a simple two step process (Equation 2). The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of 2,3-diaminonaphthalene, provided as an acidic solution, followed by sodium hydroxide which enhances the detection of the fluorescent product, 1(H)-naphthotriazole (Misko et al., 1993).



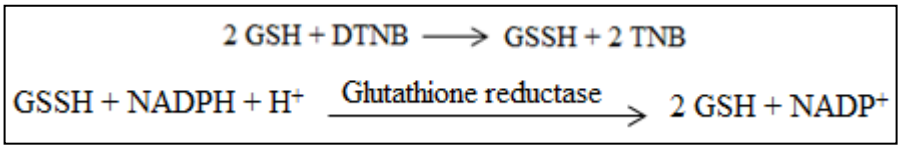
Equation 2: Scheme of nitrate/nitrite assay

2.8.3 Glutathione (GSH)

Reduced Glutathione (GSH) is the key antioxidant in animal tissues. It is present inside cells mainly in the reduced form (90-95% of the total glutathione). Intracellular GSH status appears to be a sensitive indictor of the overall health of a cell, and of its ability to resist toxic challenge. High levels of GSH in the cell may indicate pathological changes.

The glutathione assay kit from Sigma-Aldrich® was used to measure the level of total glutathione (GSSH + GSH) in the hippocampal tissue homogenate. The samples were first deproteinized with 5% 5-Sulfosalicylic Acid Solution, next centrifuged to remove the precipitated protein, and then assayed for glutathione.

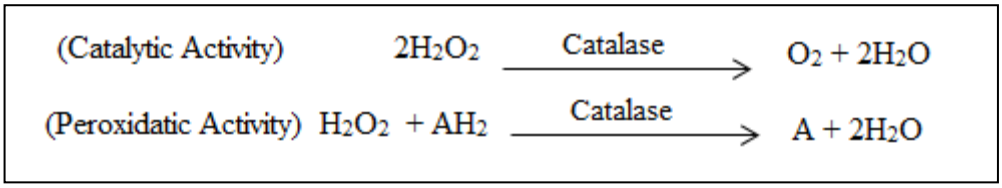
GSH was oxidized by the sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) and glutathione disulfide (GSSG). The GSSG formed was recycled to GSH by glutathione reductase in the presence of NADPH (Equation 3) and the TNB was measured spectrophotometrically (Akerboom and Sies, 1981). The plate reader was set at a wavelength of 412 nm, with kinetic reading at 1 minute interval for 5 minutes.



Equation 3: GSH assay

2.8.4 Catalase (CAT)

Catalase (CAT) is a ubiquitous antioxidant enzyme that is present in most cells. CAT is involved in the detoxification of hydrogen peroxide (H_2O_2), a reactive oxygen species, which is a toxic product. This enzyme demonstrates catalytic activity and peroxidatic activity (Equation 4).



Equation 4: Catalase activity

Cayman's Catalase Assay Kit® was employed to measure CAT activity in tissue homogenates of the hippocampus. It utilized the peroxidatic function of CAT for determination of enzyme activity (Wheeler et al., 1990). The method was based on the reaction of CAT with methanol in the presence of H_2O_2. Formaldehyde was produced and measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. It specifically formed a bicyclic heterocycle with aldehydes, which upon oxidation changed from colorless to a purple color. The absorbance was read at 540 nm wavelength.

2.8.5 Superoxide Dismutase (SOD)

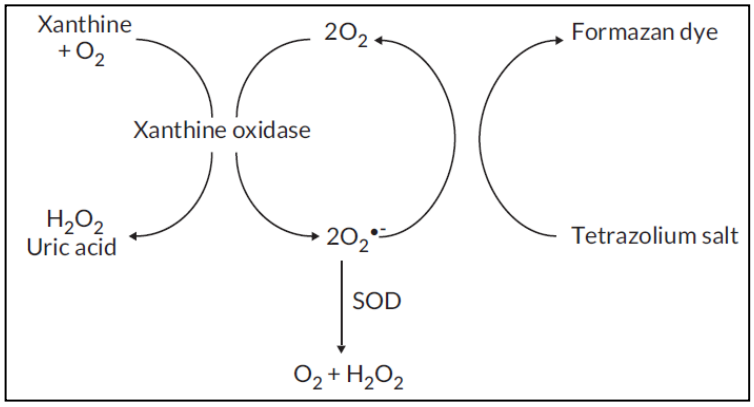
Superoxide dismutase (SOD) is a metalloenzyme that is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes and kidney. It catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide (Equation 5). The amount of SOD present

in cellular and extracellular is crucial for the prevention of diseases linked to oxidative stress.

$$2O_2^{\bullet-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

Equation 5: Superoxide Anion Dismutation

Cayman's Superoxide Dismutase Assay Kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Equation 6). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical (Sandstrom et al., 1994). The SOD assay measures all three types of SOD (Cu/Zn, Mn and Fe-SOD).



Equation 6: Scheme of the SOD assay

2.9 Growth Factor Analysis

Nerve growth factor (β-NGF) is involved in the maintenance of the sympathetic and sensory nervous systems. It exerts a number of different effects on neurons, such as development, survival, and differentiation. Neurons that fail to obtain sufficient NGF die by apoptosis. Insulin like growth factor-I (IGF-I), a

mediator of growth hormone actions, has also been shown to be an important regulator of cell metabolism, differentiation, and survival.

A DuoSet ELISA from R&D Systems assay was used for the quantitative measurement of these growth factors in hippocampal tissue homogenate (Crowther, 1995). It employed a specific antibody coated on a 96-well plate. The growth factors present in a sample was bound to the wells by the immobilized antibody. Later, biotinylated detection antibody was added followed by HRP-conjugated streptavidin. The addition of the substrate solution (1:1 Tetramethylbenzidine and H_2O_2) allowed color development in proportion to the amount of the growth factor bound. Finally, the Stop Solution (2N sulfuric acid) changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.10 Western Blotting for NFκB and AKT

The hippocampal supernatants were electrophoresed on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were then blocked in 5% non fat dry milk in PBS/Tween-20 for 1 hr at RT with gentle agitation. After blocking, the membranes were incubated with appropriate primary antibody, rabbit anti-mouse NFκB, rabbit anti-mouse AKT (Protein kinase B) or rabbit anti-mouse β-actin, overnight at 4°C. After extensive washing in PBS/Tween-20, membranes were incubated with peroxidase-conjugated secondary antibodies for 1 hr at RT. The membranes were rinsed again. Enhanced chemiluminescent Western blotting detection reagents were used for exposure according to the manufacturer's instructions. Densitometric analysis was performed using the Image J program. Results were presented as "relative densities to β-actin".

2.11 Determination of NFκB Levels by ELISA

The levels of NFκB were assessed using the commercial provided NFκB/p65 ActivELISATM Kit (Imgenex Corporation, USA) (Shi et al., 2014). Briefly, standard 96-well flat bottom NUNC-Immuno maxisorp ELISA plates (NUNC, Roskilde, Denmark) were incubated with the captured antibody at 4 °C overnight. In the next day, the plates were blocked by incubation for 1 hr at RT with 1% Bovine Serum Albumin (Sigma) in PBS. Serial dilutions of standard protein ranging from 0 to 10,000 ng/ml were applied in duplicate for the standard curve. Wells containing the standard proteins and supernatants of hippocampal tissue homogenates were incubated at 4 °C overnight as specified in the protocol. The plate was then incubated with the detecting antibody for 1 hr at RT. The Alkaline phosphatase (AKP)-Conjugated Secondary antibody as a tertiary reactant was incubated for 1 hr at RT. The p-nitrophenylphosphate Substrate was used to develop color and the absorbance was recorded at 405 nm in a plate reader within 30 min. The NFκB levels were evaluated by comparison with the regression curve for standard.

2.12 Data Presentation and Statistics

Each set of data was presented as mean value ± standard error of the mean (SEM). The one-factor analysis of variance (ANOVA) and Bonferroni post test were used to compare values within groups. Student's t-test was used to compare values between groups in behavioral tests. All tests were two-tailed, and the level of significance was set to $p < 0.05$. Graphics and calculations were performed using GraphPad PRISM version 5.0, and SPSS Version 19.0.

Chapter 3: Results

3.1 Seizure Activity

After intranasal kainic acid (KA) treatment, both wild type (Wt) and TNF-α knockout (Ko) mice manifested catatonic and staring behavior within few minutes, followed by myoclonic twitching and frequent rearing and falling. Within 20–30 min of the administration of KA, all mice displayed seizures. After the seizure activity ceased, the surviving mice of both groups assumed a hunched posture, were immobile for another few hours, and recovered in a similar way.

Seizures were rated based on the criteria described by Ben-Ari (1985) with some modifications as follows:

- 0, normal;
- 1, immobilization;
- 2, rearing and falling;
- 3, seizure for less than 1 hr;
- 4, seizure for 1-3 hr;
- 5, seizure for more than 3 hr; and
- 6, death.

The results showed that the onset of seizure activity was significantly earlier in TNF-α Ko mice compared to Wt mice. In addition, the seizures of the TNF-α Ko mice were significantly longer lasting than those of the Wt mice (Figure 6). Water-treated Wt and TNF-α Ko mice did not show any symptoms.

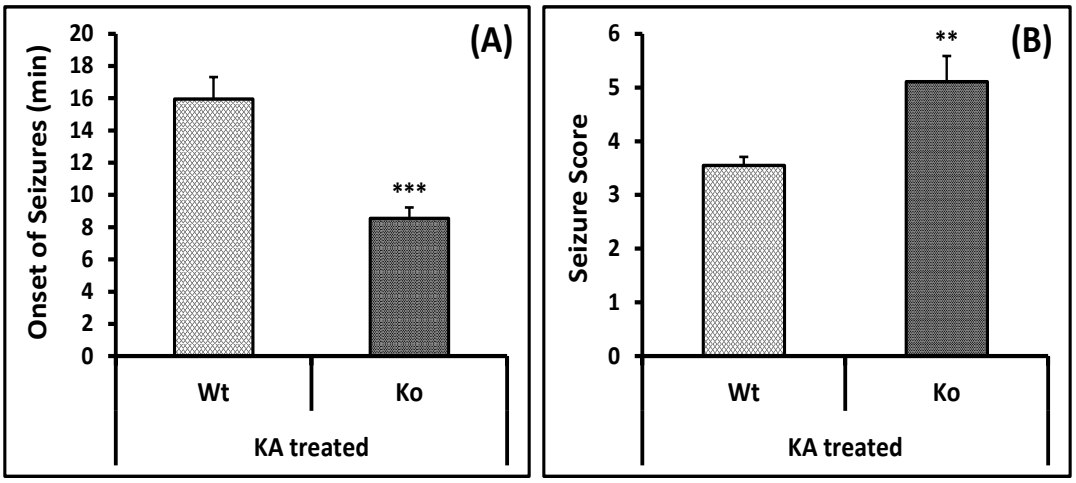


Figure 6: Onset of Seizures and Seizure Scores for Wt and TNF-α Ko mice after intranasal administration of KA

Data represent the Mean ± SEM, (n = 50), ** Significant from Wt at P<0.01, * Significant from Wt at P<0.001.**

3.2 Behavioral Tests

Both TNF-α Ko and Wt mice were exposed to three behavioral tests before and after treatment with either water or KA at different time points. These behavioral tests are Elevated Plus Maze (EPM), Open Field test and Y-Maze. Initially, water treated Wt and TNF-α Ko mice did not show any significant behavioral changes in the tests and the results were comparable to those of untreated mice.

3.2.1 Elevated Plus-Maze

In Elevated Plus-Maze (EPM), the following parameters were recorded for each mouse over a period of 5 min: 1) number of entries into open arms; 2) number of entries into closed arms; 3) time spent in open arms; 4) time spent in closed arms; 5) number of observations of head dipping over sides of open arms; 6) number of risk assessment behaviors; and 7) number of observations of feces in 5 min (Figure

7). This test is performed for all mice 3 days before KA treatment and 5, 15 and 30 days after KA treatment. Usually normal mice prefer to stay more in the closed arms.

Following KA treatment, Both Wt and TNF-α Ko mice showed a significant changed behavior compared with those observed before KA treatment. They showed increased frequency and duration in open arms and decreased frequency in closed arms specifically at 5 and 15 days post KA treatment. At 30 days post KA treatment, TNF-α Ko mice significantly stayed more time in the open arms, while the Wt mice spent more time in closed arms and showed lower frequency and duration in open arms (Fig. 7 A-D). At all time points, both Wt and TNF-α Ko mice showed decreased head drops in open arms (a measure of exploration as well) and risk assessment behavior compared with those observed before KA treatment (Fig. 7 E-F). However, TNF-α Ko mice showed significantly decreased counts of risk assessment 30 days post KA treatment compared to Wt mice. The number of feces during the test period (5 min) is an additional measure for the degree of anxiety that was significantly increased in TNF-α Ko mice 5, 15 and 30 days post KA treatment compared to respective KA treated Wt groups (Fig. 7G). Taken together, these results showed changed risk assessment performance and decreased exploration in the TNF-α Ko mice on the EPM compared to KA treated Wt mice, specifically at 30 days post KA treatment.

3.2.2 Open Field

The open-field test is performed for all mice before KA treatment and 3, 5, 15 and 30 days after KA treatment. Both locomotion and rearing are recorded for all mice over a period of 9 min.

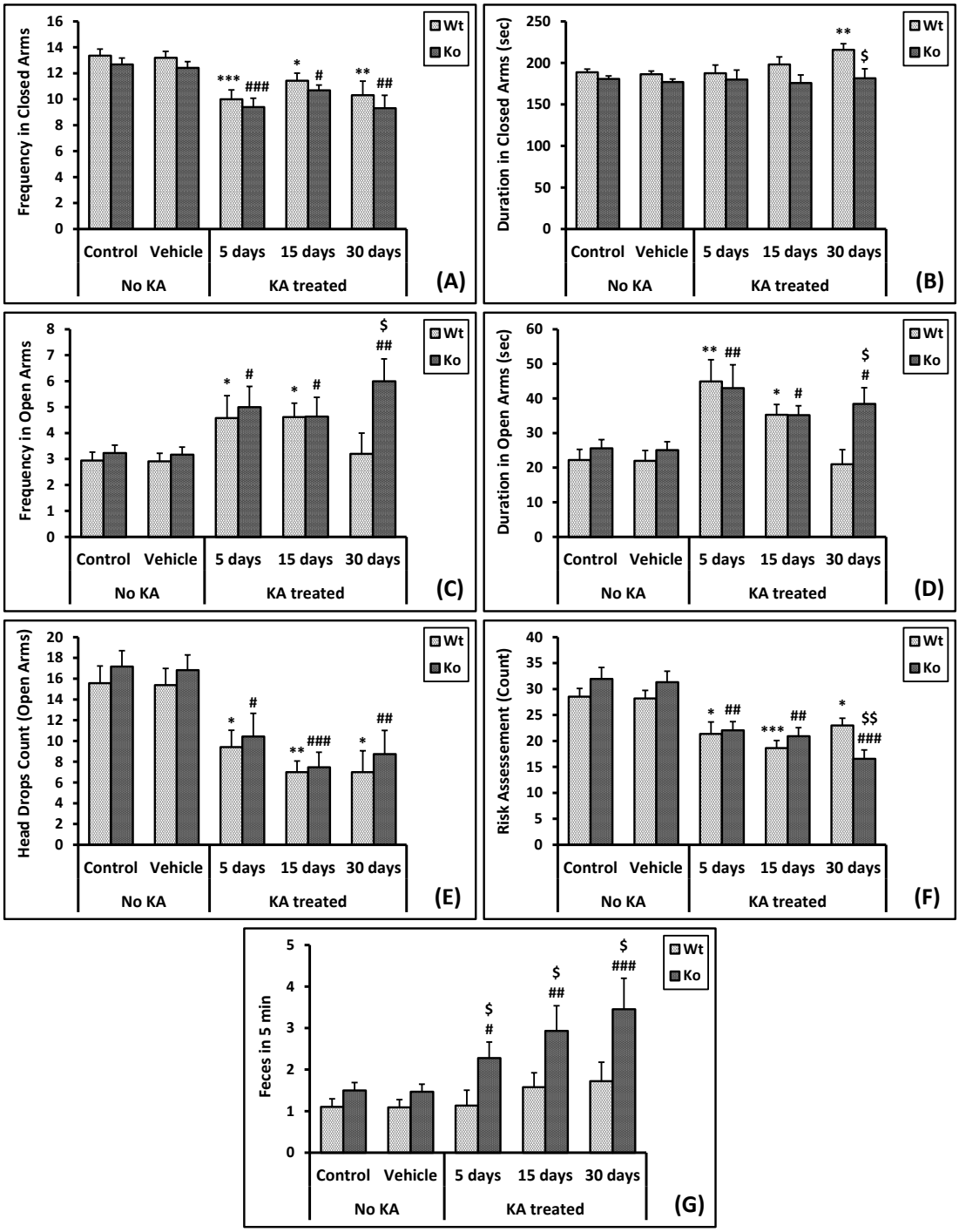


Figure 7: Elevated Plus Maze Parameters for Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 15-22), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05, $$ Significant from respective Wt KA treated at P<0.01.**

KA treatment resulted in a state of hyperactivity in Wt and TNF-α Ko mice in the form of increased locomotion and rearing counts compared with those observed before KA treatment. The highest level of locomotion activity was observed 3 days post KA treatment. TNF-α Ko mice showed significantly more locomotion activity compared with KA treated Wt mice at 3 days and 30 days post KA treatment (Figure 8). Both TNF-α Ko and Wt mice showed increased exploratory activity as evident by increased rearing count at 3, 5, 15, and 30 days post KA treatment compared with the rearing activity observed before KA treatment (Figure 9). The highest level recorded for rearing activity was 3 days post KA treatment.

3.2.3 Y-Maze

In a Y-maze test, the number of arm entries as well as the percent of successful alternation between arms were recorded for all mice before KA treatment and 3, 5, 15 and 30 days after KA treatment. Both KA treated TNF-α Ko and Wt mice showed more arm entries compared with those observed before KA treatment (Figure 10). This observation is in line with the hyperactivity observed in the Open Field test. Similarly, the highest arm entries count was observed in both groups 3 days post KA treatment (Fig. 10 A-C). On the other hand, both KA treated TNF-α Ko and Wt mice exhibited decreased successful alternation at 3 and 5 days post KA treatment compared to those observed before KA treatment. At 15 and 30 days post KA treatment, TNF-α Ko mice still showed decreased successful alternation while Wt mice showed improved levels of alternation that is comparable to those obtained before KA treatment. Percent alternation of TNF-α Ko mice at 30 days post KA treatment is significantly lower compared to the respective KA treated Wt mice (Fig. 10 D).

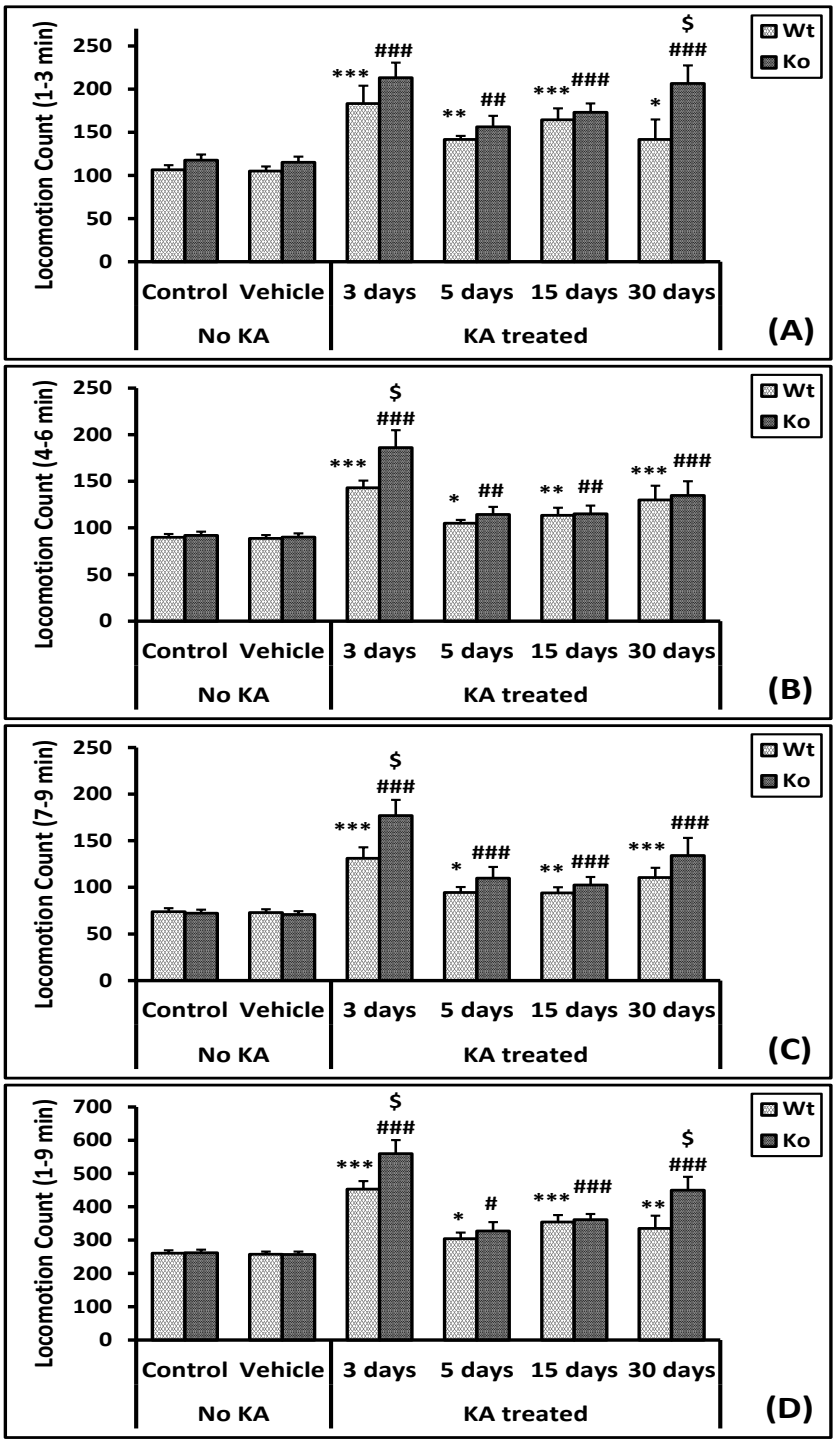


Figure 8: Locomotion count in Open Field Test for Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 15-22), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05.**

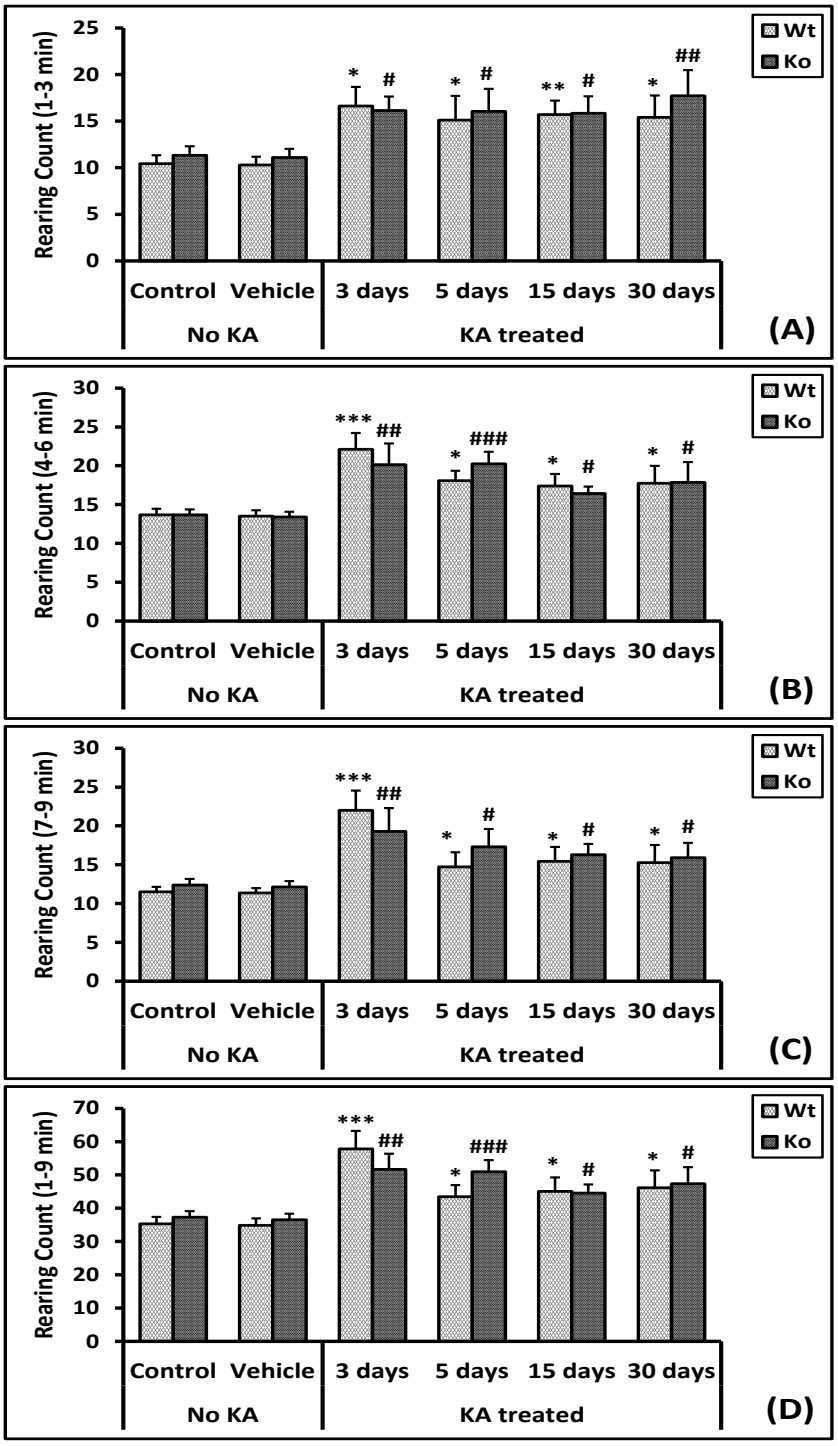


Figure 9: Rearing count in Open Field Test for Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 15-22), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001.**

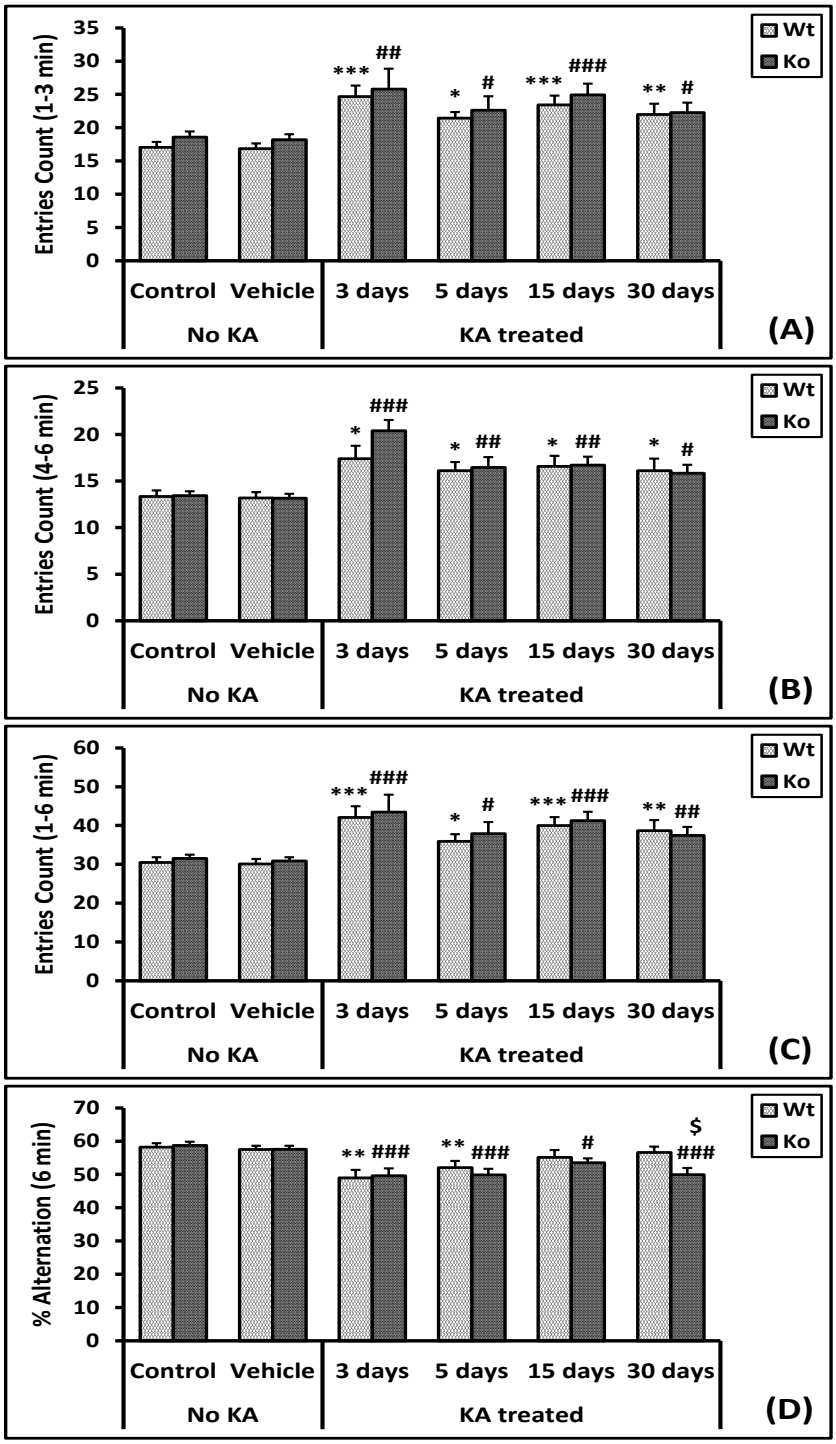


Figure 10: Y-Maze test for Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 15-22), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05.**

3.3 Hippocampal Cytokine Levels

Cytokine levels were measured in the hippocampal supernatants of control untreated, water treated and KA treated TNF-α Ko and Wt mice at different time points (0.5 hr, 4 hr, 1, 3, 5, 15 and 30 days). Water treated Wt and TNF-α Ko mice showed comparable values to those of control untreated mice.

3.3.1 TNF-α

Wt mice exposed to KA treatment exhibited a significant increase in the levels of TNF-α in the hippocampus 0.5 and 4 hr as well as 1, 3 and 5 days post KA treatment compared to their respective untreated controls. The hippocampal TNF-α returned to normal levels at 15 and 30 days post KA treatment (Figure 11).

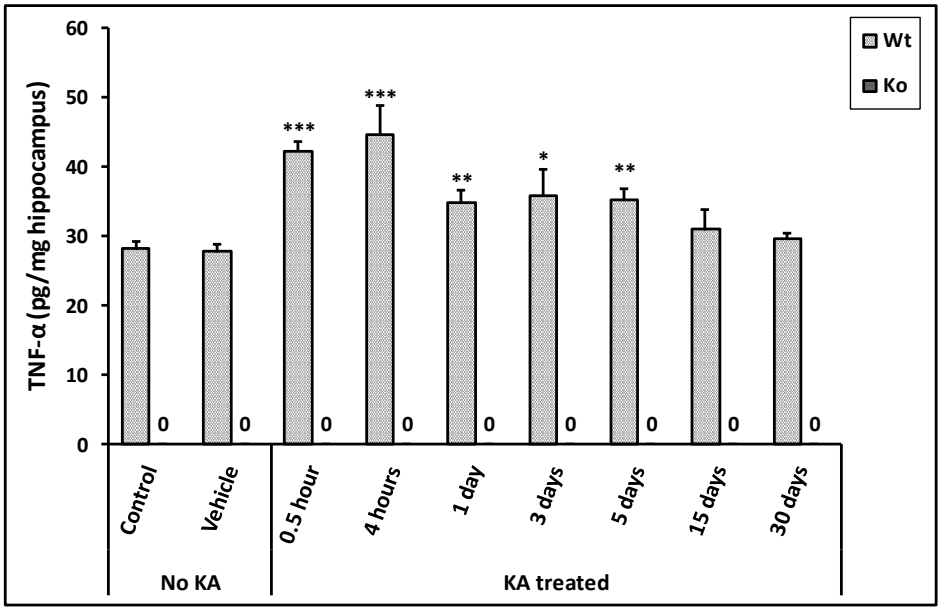


Figure 11: Hippocampal TNF-α levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001.**

3.3.2 IL-6

TNF-α Ko and Wt mice exposed to KA treatment exhibited a significant increase in the levels of IL-6 in the hippocampus 0.5 and 4 hr post KA treatment compared to their respective untreated controls. During this period, most of the mice exhibited seizures. The hippocampal IL-6 levels returned to normal levels at 1, 3, 5, 15 and were significantly decreased at 30 days post KA treatment in TNF-α Ko mice (Figure 12).

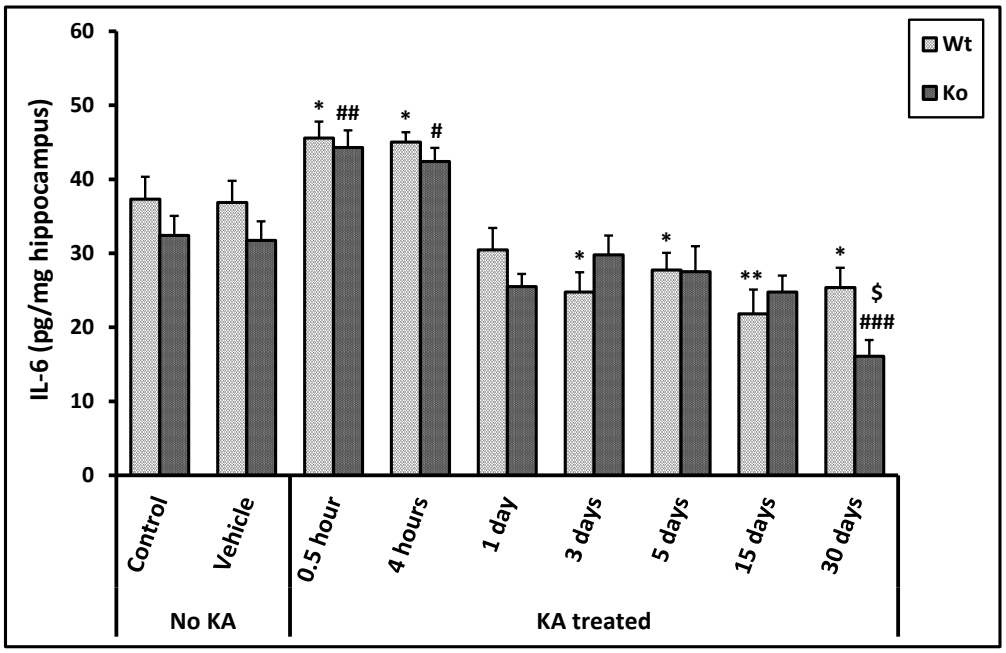


Figure 12: Hippocampal IL-6 levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05.**

3.3.3 IL-1β

Similar to hippocampal IL-6 levels, TNF-α Ko and Wt mice exposed to KA treatment exhibited a significant increase in the levels of IL-1β in the hippocampus 0.5 and 4 hr post KA treatment compared to their respective untreated controls. During this period, most of the mice exhibited seizures. The hippocampal IL-1β levels returned to normal levels at 1, 3, 5, 15 and 30 days post KA treatment (Figure 13).

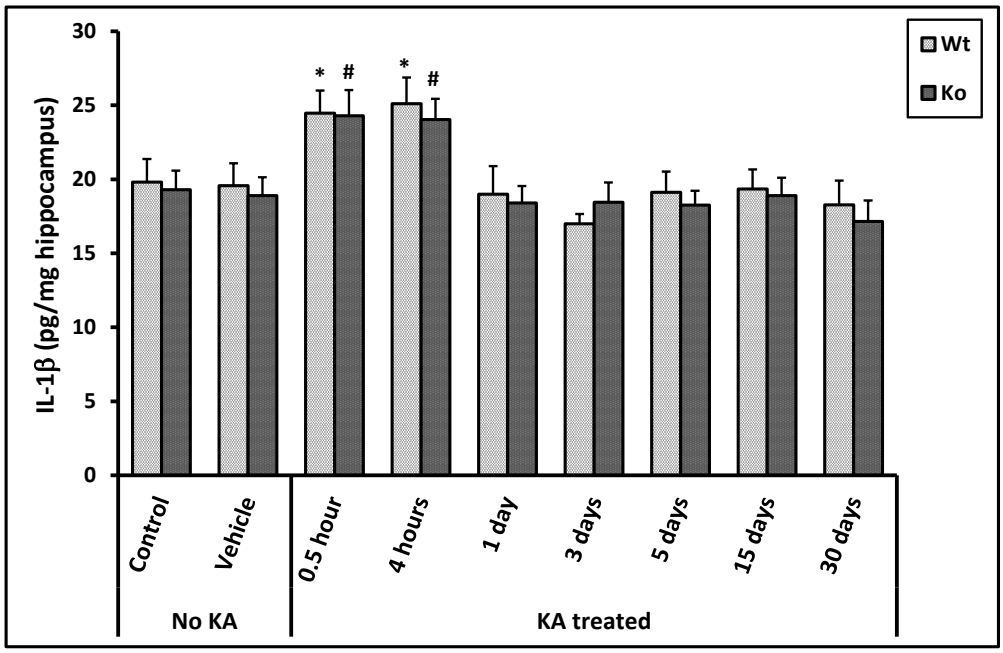


Figure 13: Hippocampal IL-1β levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, # Significant from Ko Control at P<0.05.**

3.3.4 IL-12

An elevation in the levels of hippocampal IL-12 was observed at 0.5 and 4 hr post KA treatment in KA treated TNF-α Ko and Wt mice compared to the respective untreated controls. This increase is followed by a significant decrease that happened earlier in TNF-α Ko mice (1 day post KA) compared to Wt mice (3 days post KA). At 5, 15 and 30 days post KA; the levels of IL-12 in Wt mice were comparable or significantly less than the normal levels. The IL-12 levels in TNF-α Ko mice returned to normal levels at 3 and 5 days post KA treatment, however they were significantly lower than their respective untreated controls and KA treated Wt mice at 15 and 30 days post KA treatment (Figure 14).

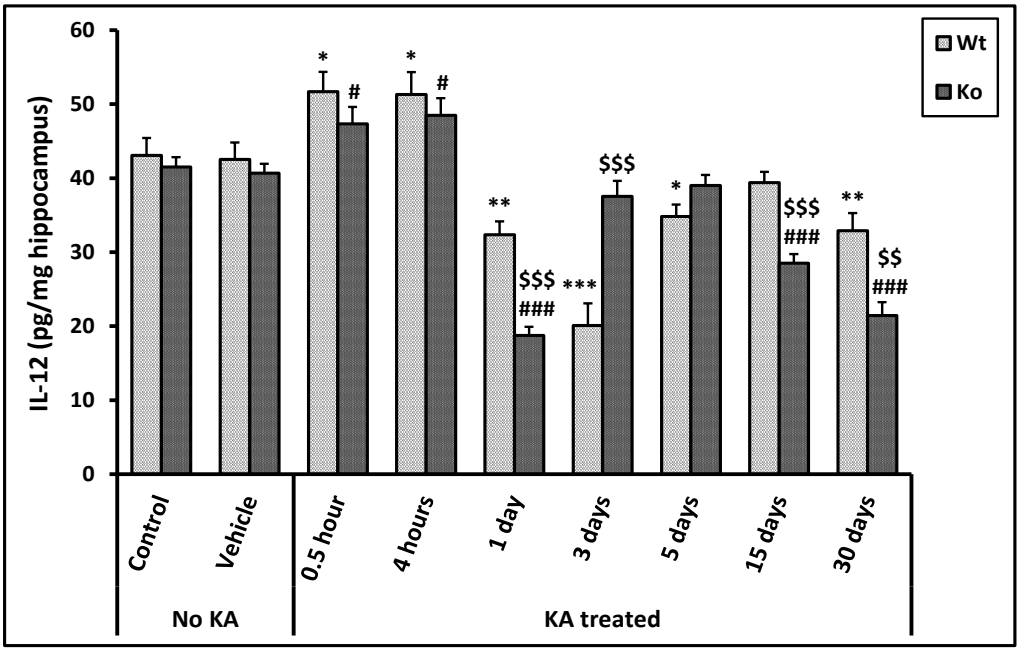


Figure 14: Hippocampal IL-12 levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ### Significant from Ko Control at P<0.001, $$ Significant from respective Wt KA treated at P<0.01, $$$ Significant from respective Wt KA treated at P<0.001.**

3.3.5 IL-10

The basal levels of hippocampal IL-10 were significantly lower in control and water treated TNF-α Ko mice compared to control and water treated Wt mice. Half an hour post KA treatment, TNF-α Ko mice showed a significant elevation in the levels of hippocampal IL-10 compared to their respective untreated controls. The levels of hippocampal IL-10 returned to normal levels starting from 4 hr post KA to 1, 3, 5 and 15 days but showed a significant decrease at 30 days post KA treatment compared to their untreated controls and KA treated Wt mice. However, the Wt mice showed significantly lower levels of hippocampal IL-10 starting from 4 hr post KA to 1, 3, 5, 15 and 30 days compared to their respective untreated controls (Figure 15).

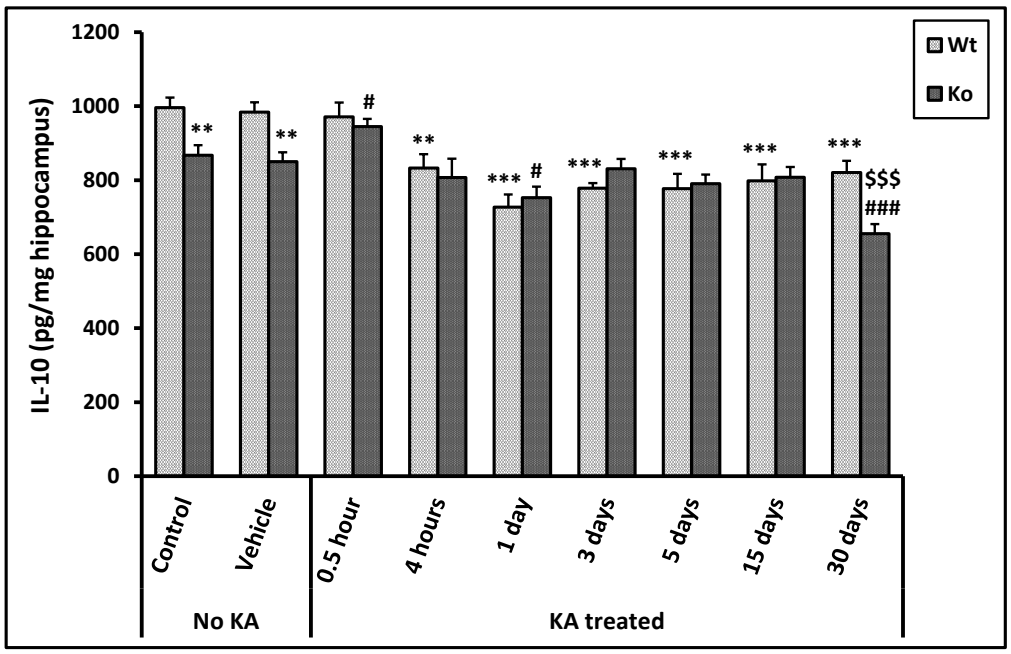


Figure 15: Hippocampal IL-10 levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
**** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ### Significant from Ko Control at P<0.001, $$$ Significant from respective Wt KA treated at P<0.001.**

3.4 Oxidative Stress Markers in Hippocampus

Oxidative stress markers were measured in the hippocampal supernatants of control untreated, water treated and KA treated TNF-α Ko and Wt mice at different time points (0.5 hr, 4 hr, 1, 3, 5, 15 and 30 days). These markers include: Malondialdehyde (MDA), glutathione (GSH), Catalase (CAT), superoxide dismutase (SOD) and nitric oxide (NO). Water treated Wt and TNF-α Ko mice showed comparable values to those of control untreated mice.

3.4.1 Malondialdehyde (MDA)

Lipid peroxidation is used as an indicator of oxidative stress in cells and malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. KA treatment resulted in production of reactive oxygen species and oxidative stress. Both KA treated TNF-α Ko and Wt mice showed elevated hippocampal MDA levels at all time points compared to their respective untreated controls. However, KA treated TNF-α Ko mice showed significantly high MDA levels 1 day post KA compared to the respective KA treated Wt mice indicating more oxidative stress in TNF-α Ko mice at this time point (Figure 16).

3.4.2 Nitric Oxide (NO)

Wt mice showed significantly higher levels of NO at 0.5 and 4 hr following KA administration. TNF-α Ko mice exhibited significantly increased production of NO at 0.5 hr post KA, reaching its peak value 4 hr post KA and remaining significantly elevated at 1, 3 and 5 days and returned to normal levels at 15 and 30

days post KA compared with their untreated controls and respective KA treated Wt mice (Figure 17).

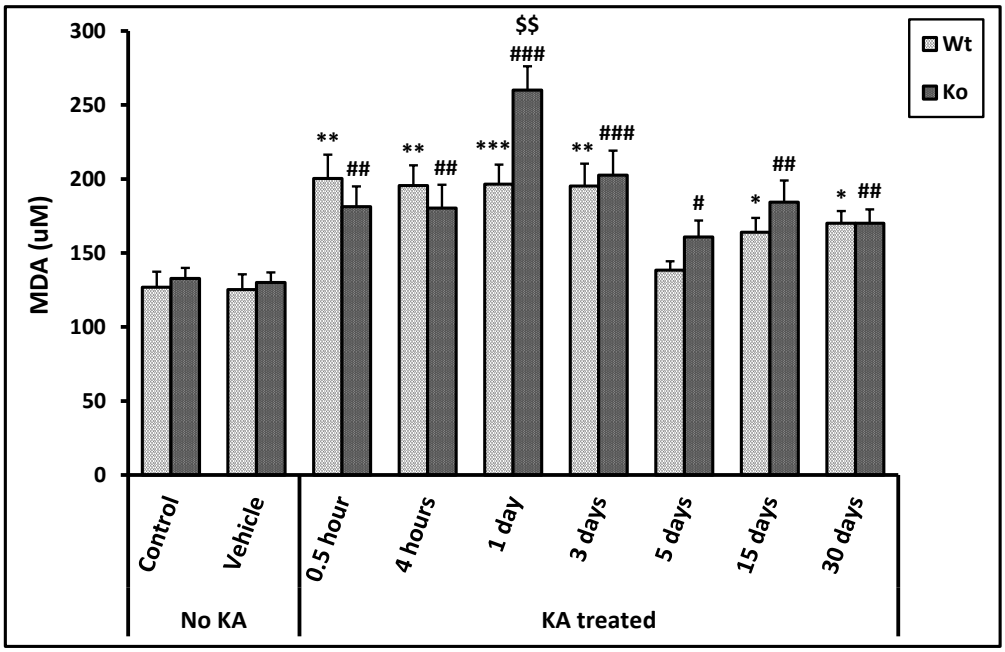


Figure 16: Hippocampal MDA levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $$ Significant from respective Wt KA treated at P<0.01.**

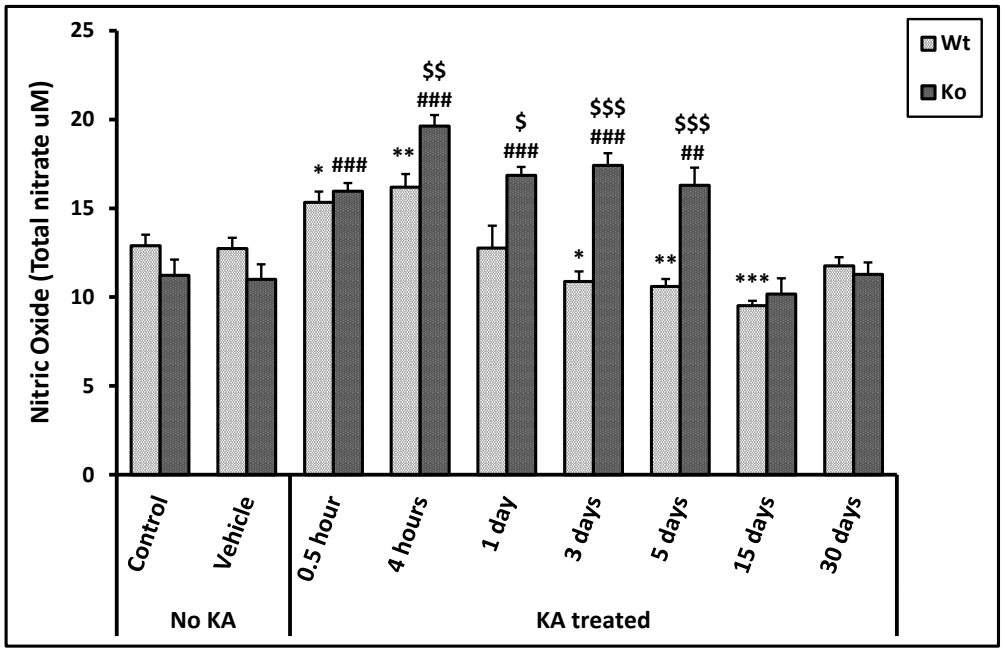


Figure 17: Hippocampal Nitric Oxide levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05, $$ Significant from respective Wt KA treated at P<0.01, $$$ Significant from respective Wt KA treated at P<0.001.**

3.4.3 Glutathione (GSH)

Glutathione (GSH) is one of the defensive mechanisms against oxidative stress. The basal levels of hippocampal GSH were significantly higher in control and water treated TNF-α Ko mice compared to control and water treated Wt mice. At 1 and 3 days after KA administration, Wt mice showed significantly elevated hippocampal GSH levels compared to their untreated controls. This elevation in GSH levels represented a defensive mechanism against increased oxidative stress, but it did not persist for more than 3 days after which the GSH levels declined significantly and were nearly depleted at 5, 15 and 30 days post KA. On the other hand, TNF-α Ko mice did not show elevation of hippocampal GSH levels post KA treatment.

Hippocampal GSH levels in TNF-α Ko mice were significantly reduced starting from 0.5 hr to 4 hr and 1 day post KA. It was further reduced significantly and nearly depleted at 3, 5, 15 and 30 days post KA (Figure 18).

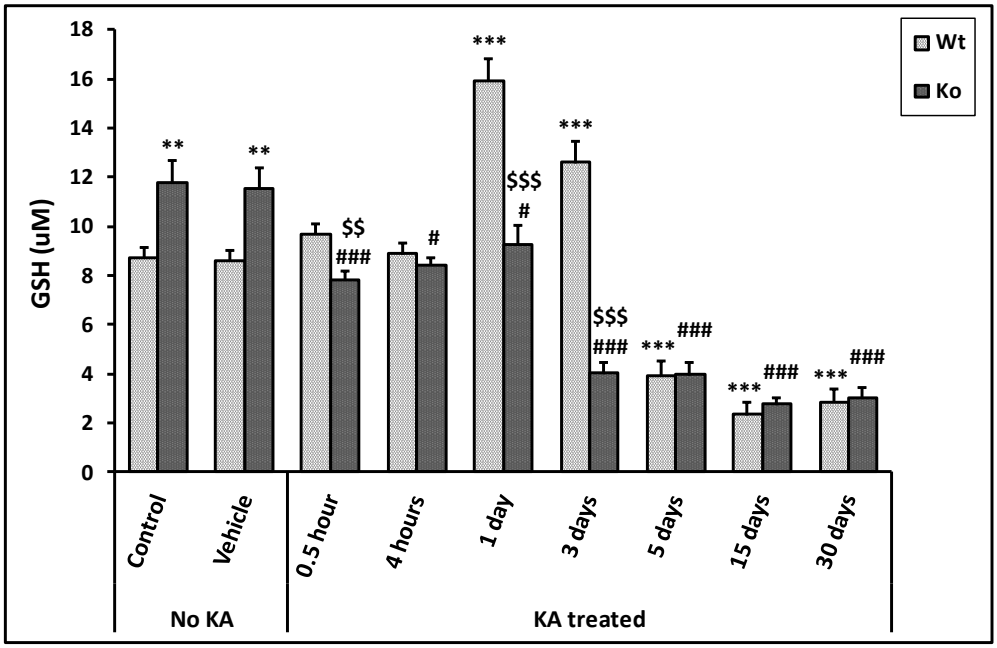


Figure 18: Hippocampal GSH levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
**** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ### Significant from Ko Control at P<0.001, $$ Significant from respective Wt KA treated at P<0.01, $$$ Significant from respective Wt KA treated at P<0.001.**

3.4.4 Catalase (CAT)

Catalase (CAT) is an antioxidant enzyme that is present in most cells as one of the defensive mechanisms against oxidative stress. The basal activity of hippocampal CAT were significantly higher in control and water treated TNF-α Ko mice compared to control and water treated Wt mice. In KA treated Wt mice, hippocampal CAT activity were significantly elevated only at 5 days post KA

treatment compared to their untreated controls. TNF-α Ko mice showed a significant initial decrease at 0.5 and 4 hr post KA treatment followed by a significant increase at 1, 3 and 5 days post KA treatment compared to their untreated controls and respective KA treated Wt mice, possibly as a defensive effort against oxidative stress. Finally, hippocampal CAT activity significantly declined again 30 days post KA compared to their untreated controls and respective KA treated Wt mice (Figure 19)

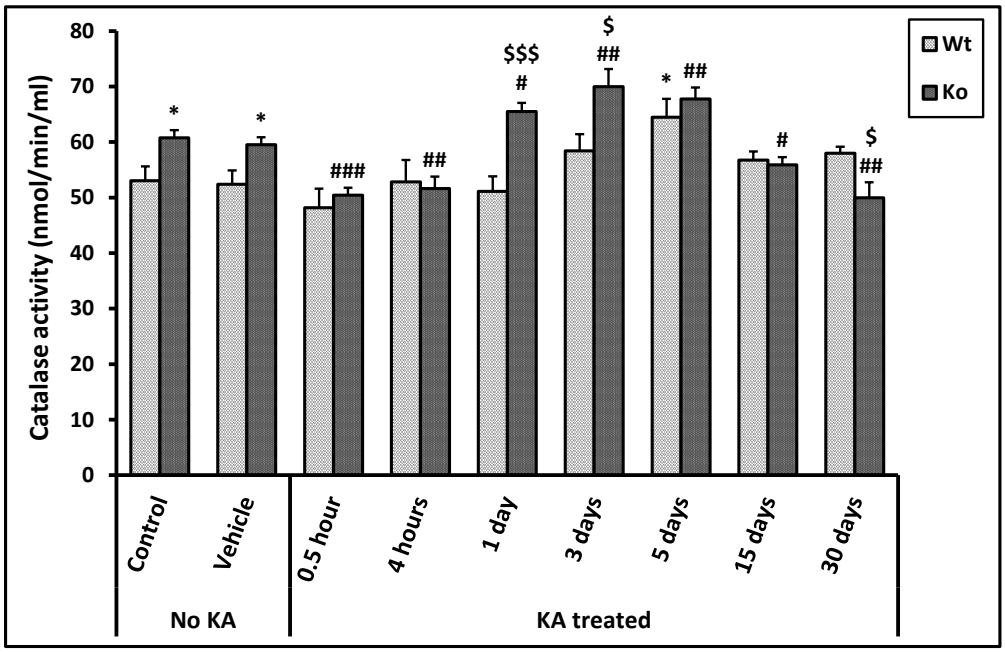


Figure 19: Hippocampal Catalase Activity in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05, $$$ Significant from respective Wt KA treated at P<0.001.**

3.4.5 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is an antioxidant enzyme that is present in most cells as one of the defensive mechanisms against oxidative stress. KA treated Wt mice showed significantly higher hippocampal SOD activity 4 hr and 1 day post KA treatment compared to their untreated controls, while TNF-α Ko mice showed significantly higher hippocampal SOD activity at 0.5 hr, 3 days and 30 days post KA treatment compared to their untreated controls (3 and 30 days) and respective KA treated Wt mice (0.5 hr, 3 and 30 days) (Figure 20).

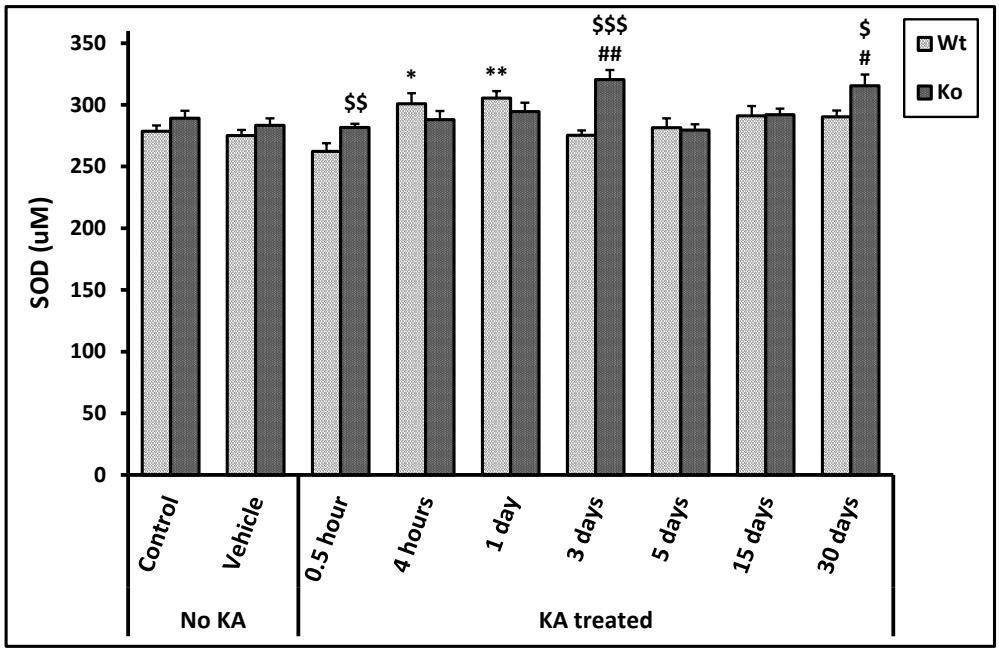


Figure 20: Hippocampal SOD levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, $ Significant from respective Wt KA treated at P<0.05, $$$ Significant from respective Wt KA treated at P<0.001.**

3.5 Hippocampal Growth Factors

β-NGF and IGF-I were measured in the hippocampal supernatants of control untreated, water treated and KA treated TNF-α Ko and Wt mice at different time points (0.5 hr, 4 hr, 1, 3, 5, 15 and 30 days). Water treated Wt and TNF-α Ko mice showed comparable values to those of control untreated mice.

3.5.1 Nerve Growth Factor (β-NGF)

β-NGF is involved in the maintenance of the sympathetic and sensory nervous systems. KA treated Wt mice did not show any changes in the levels of hippocampal β-NGF at all time points, while TNF-α Ko mice showed a significant elevation in hippocampal β-NGF levels at 0.5 hr, 4 hr and 1 day after which it returned to normal levels at 3, 5, 15 and 30 days post KA treatment (Figure 21).

3.5.2 Insulin-like Growth Factor (IGF-I)

Insulin like growth factor-I (IGF-I) is a mediator of growth hormone actions and it has been shown to be an important regulator of cell metabolism, differentiation, and survival. The basal levels of hippocampal IGF-I were significantly lower in control and water treated TNF-α Ko mice compared to control and water treated Wt mice. Upon exposure to KA, the hippocampal IGF-I levels of the TNF-α Ko mice were significantly reduced starting at 0.5 hr compared to their untreated controls and/or respective KA treated Wt mice, then IGF-I levels returned to normal levels at 1 day and 3 days post KA before declining again at 5, 15 and 30 days post KA. On the other hand, KA treated Wt mice showed a transient significant decrease in hippocampal levels of IGF-I starting within 4 hr post KA treatment

which returned to normal levels at 1 day post KA. Moreover, the levels of IGF-I were significantly reduced at 3, 5, 15 and 30 days post KA but not as much reduced as in TNF-α Ko mice (Figure 22).

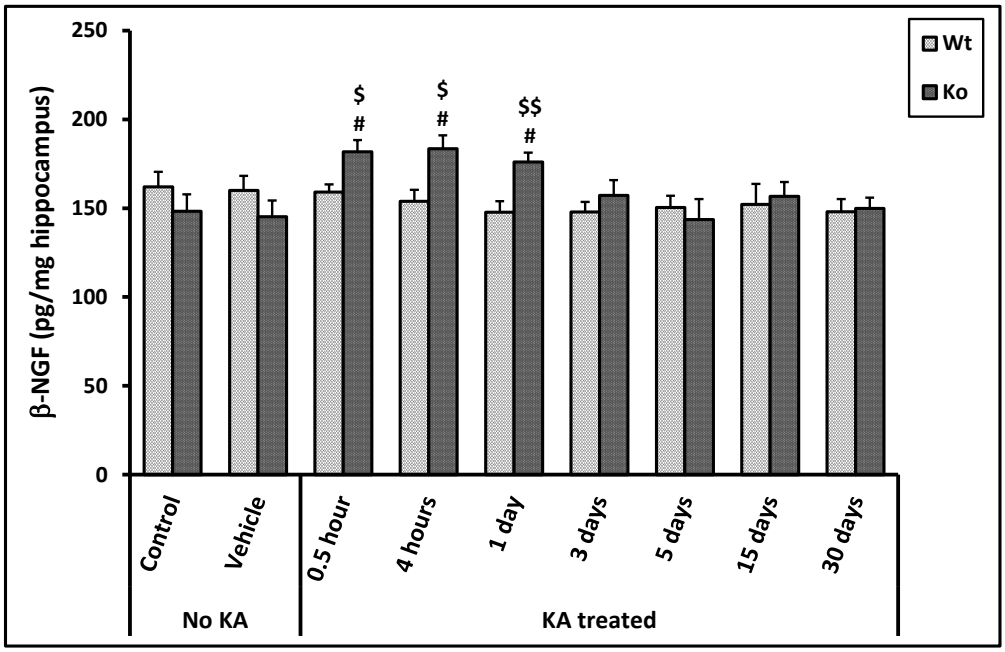


Figure 21: Hippocampal β-NGF levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
 # Significant from Ko Control at P<0.05, $ Significant from respective Wt KA treated at P<0.05, $$ Significant from respective Wt KA treated at P<0.01.

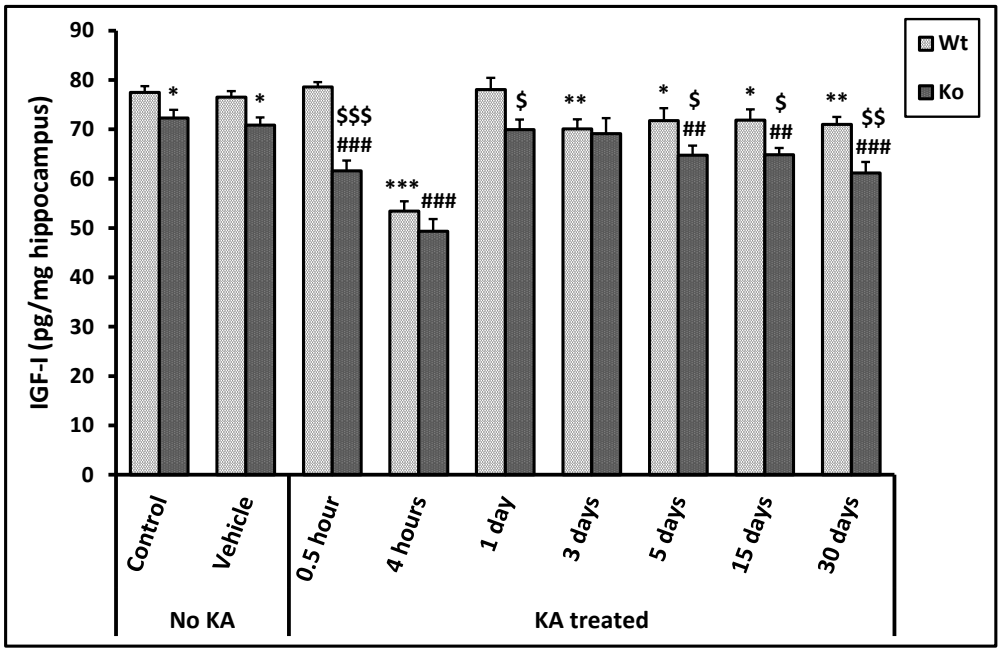


Figure 22: Hippocampal IGF-I levels in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05, $$ Significant from respective Wt KA treated at P<0.01, $$$ Significant from respective Wt KA treated at P<0.001.**

3.6 Immunohistochemistry

Hippocampal neurodegeneration, microglial activation and astrogliosis were all examined in the hippocampi of control untreated, water treated and KA treated TNF-α Ko and Wt mice at different time points (0.5 hr, 4 hr, 1, 3, 5, 15 and 30 days) by immunohistochemistry. Water treated Wt and TNF-α Ko mice showed comparable results to those of control untreated mice. Figure 23 shows a coronal section of mouse brain indicating different areas of the hippocampus (CA = Cornu Ammonis).

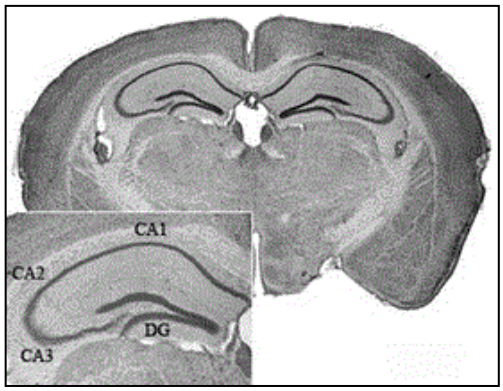


Figure 23: Coronal section of mouse brain showing different areas of the hippocampus

3.6.1 Hippocampal Neurodegeneration

Hippocampal neurodegeneration was observed by FJB staining and loss of NeuN positive cells in order to detect the time at which neurons started to die, the affected areas in the hippocampus and the extent of neurodegeneration. Using FJB staining, few degenerating neurons were observed as early as 1 day post KA in TNF-α Ko mice. Three days after KA administration, selective hippocampal neurodegeneration was observed in the CA3 area of KA treated TNF-α Ko mice. In this group, neurodegeneration was also found in the CA1 regions of some of the mice. In comparison, Wt mice showed mild neurodegeneration only in CA3 area (Figure 24). Figure 25 showed representative images for neurodegeneration in the hippocampus 5 days after KA treatment using NeuN staining. Following hippocampal neurodegeneration at different time points, it was found that more neurons were lost with time and the neuronal loss was more significant in KA treated TNF-α Ko mice compared to KA treated Wt mice as demonstrated by obvious disruption in the neuronal cell layers, decreased number and/or loss of NeuN positive cells (Figure 26) and (Figure 27). Almost 95% of TNF-α Ko mice showed CA3

lesion with variable degrees of neuronal loss, while 75% of Wt mice showed CA3 lesions. CA1 lesions were observed only in 54% of TNF-α Ko mice, and it is usually accompanied with CA3 lesion. Rarely, some Ko mice showed only CA1 lesion.

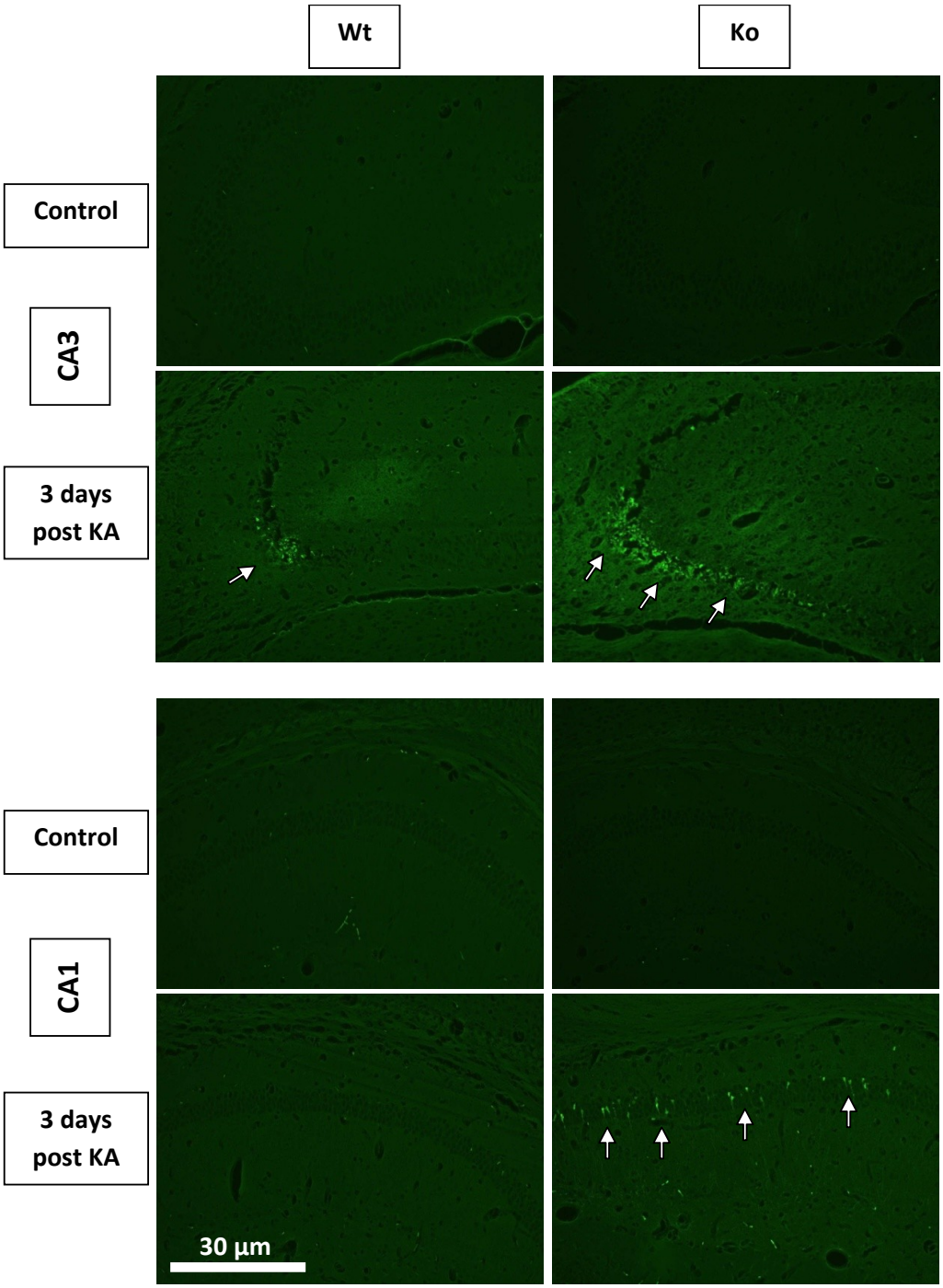


Figure 24: Selective hippocampal neurodegeneration (FJB staining) in Wt and TNF-α Ko mice before and 3 days after KA treatment

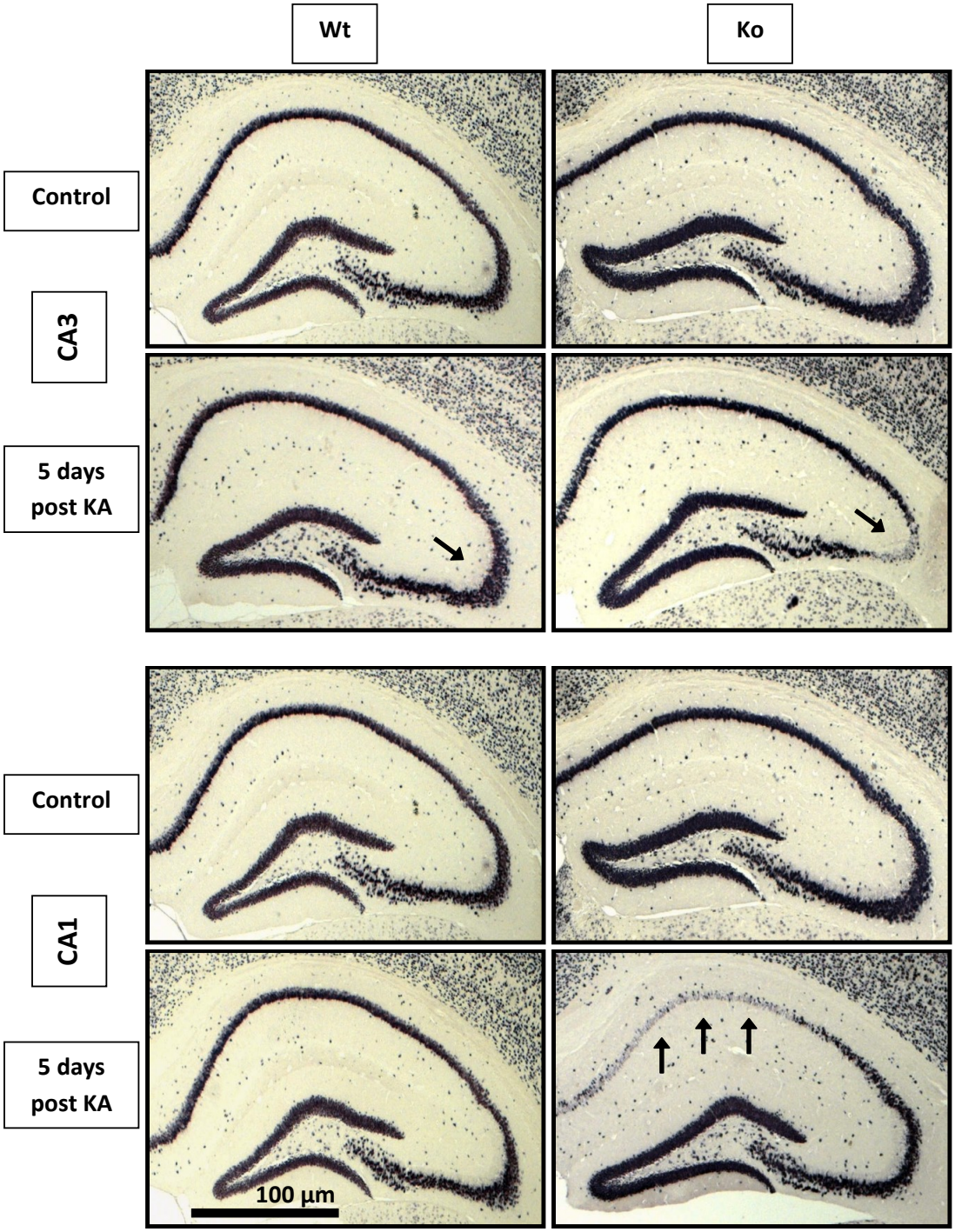


Figure 25: Selective hippocampal neurodegeneration (loss of NeuN positive cells) in Wt and TNF-α Ko mice before and 5 days after KA treatment

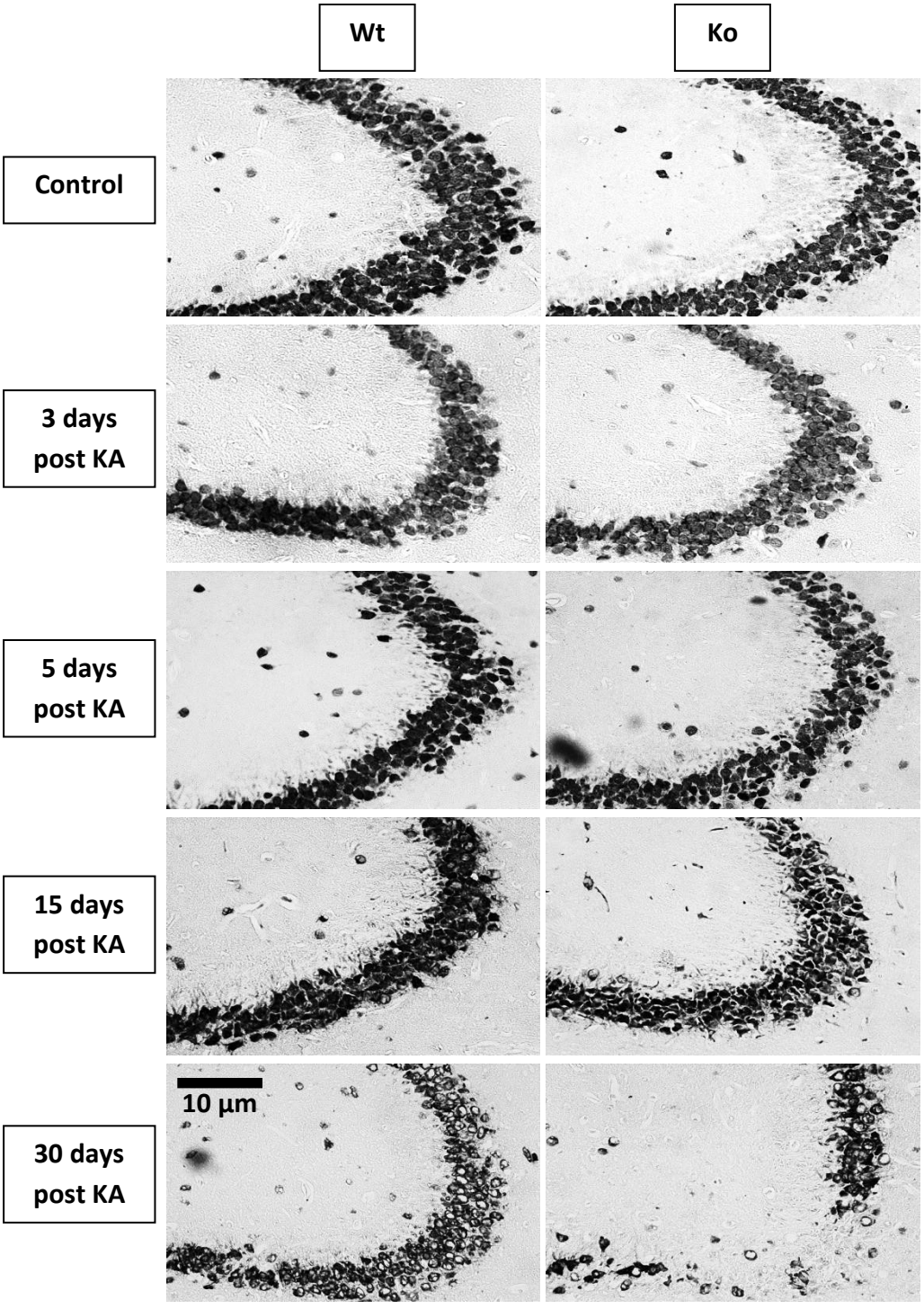


Figure 26: Hippocampal neurodegeneration in CA3 area (loss of NeuN positive cells) in Wt and TNF-α Ko mice before and after KA treatment

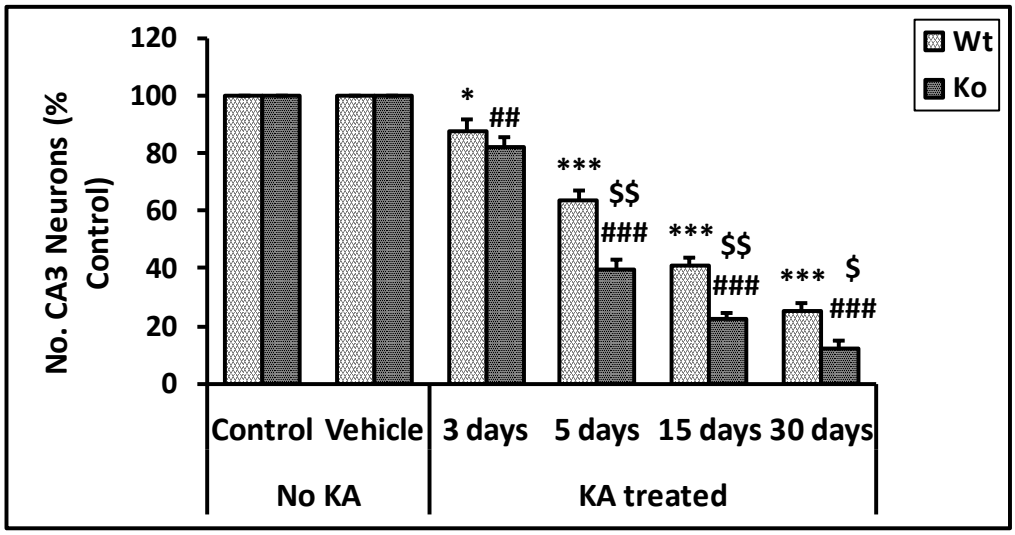


Figure 27: Semi-quantitative counting of hippocampal neurons in the CA3 area (NeuN positive cells) in Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 7-9), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, *** Significant from Wt Control at P<0.001, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05, $$ Significant from respective Wt KA treated at P<0.01.**

3.6.2 Microglial activation

Microglial activation (Iba-1 positive cells) was investigated at all time points after KA treatment by immunohistochemistry. Microglia were activated earlier in KA treated TNF-α Ko mice (0.5 hr and 4 hr post KA) compared to KA treated Wt mice. With time, the intensities of the activated microglia increased gradually in both TNF-α Ko and Wt mice, however the intensities of the activated microglia of KA treated TNF-α Ko mice were significantly greater than the intensities of KA treated Wt mice at all time points. At the late time points of 15 and 30 days post KA, clusters of microglia were found surrounding and invading the neuronal cell layers (Figure 28).

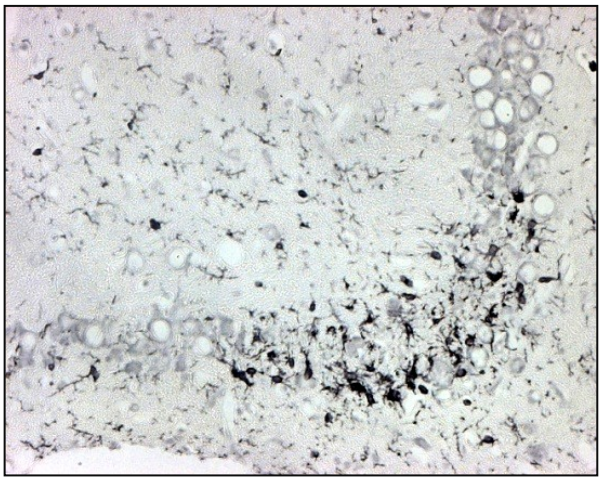


Figure 28: Activated microglia clusters invading neuronal cell layers in the CA3 area of the hippocampus of TNF-α Ko mice 30 days post KA

Microglial activation was evident in all areas of the hippocampus but with time microglia tended to be more concentrated and localized close to areas of neurodegeneration specifically neurons in the CA3 area. Figure 29 shows representative images for microglia in the CA3 area of the hippocampus at different time points for both TNF-α Ko and Wt mice. Figure 30 shows a semi-quantitative counting of microglia in the CA3 area (Iba-1 positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment.

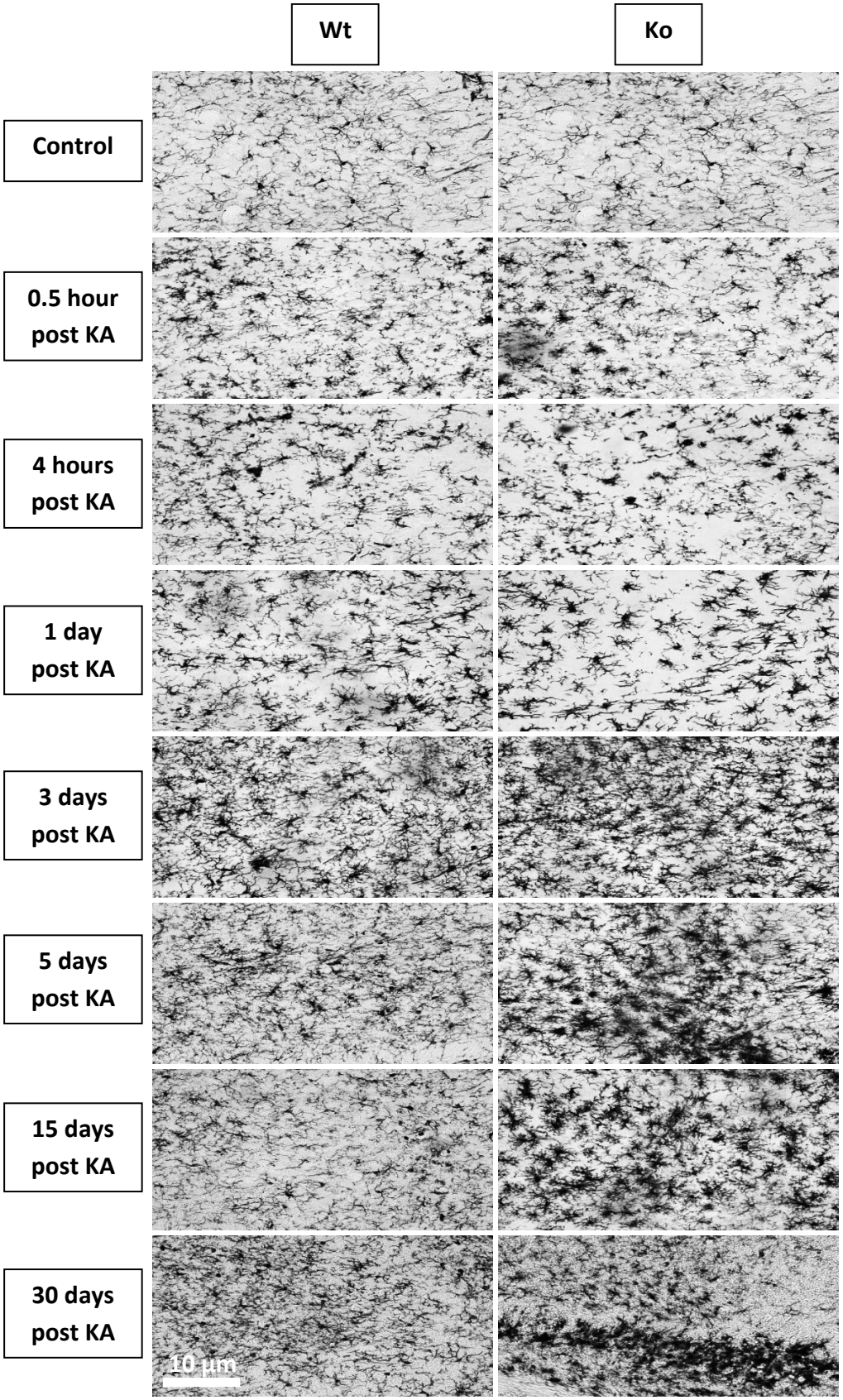


Figure 29: Microglial activation (Iba-1 positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment

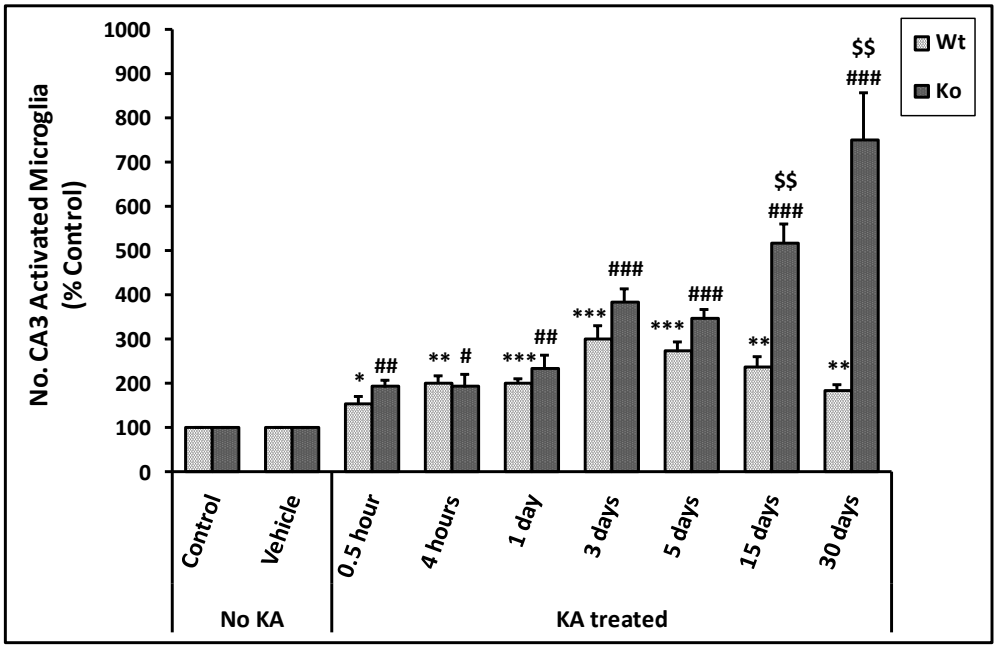


Figure 30: Semi-quantitative counting of microglia in the CA3 area (Iba-1 positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 7-9), Vehicle = Distilled Water
*** Significant from Wt Control at P<0.05, ** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, # Significant from Ko Control at P<0.05, ## Significant from Ko Control at P<0.01, ### Significant from Ko Control at P<0.001, $$ Significant from respective Wt KA treated at P<0.01.**

3.6.3 Astrogliosis

Astrogliosis (GFAP expression) was examined at all time points after KA treatment by immunohistochemistry. Usually, astrocytes are activated following microglial activation. Similar to microglia, astrogliosis occurred earlier in KA treated TNF-α Ko mice (1-3 days post KA) compared to KA treated Wt mice. With time, the intensities of astrogliosis increased gradually in both TNF-α Ko and Wt mice, however the intensities of astrogliosis of KA treated TNF-α Ko mice were significantly greater than intensities of KA treated Wt mice at all time points. Like microglia, at late time points (15 and 30 days), clusters of astrocytes were found surrounding and invading the neuronal cell layers. Astrogliosis occurred in all areas

of the hippocampus but with time astrocytes became more concentrated and localized close to areas of neurodegeneration specifically neurons in the CA3 area. Figure 31 showed a representative image for the activated astrocytes invading the neuronal cell layers in the CA3 area. Figure 32 shows representative pictures for the distribution of activated astrocytes in the hippocampus at different time points for both TNF-α Ko and Wt mice, with more focus on the CA3 area (Figure 33). Figure 34 shows a semi-quantitative counting of astrocytes in the CA3 area (GFAP positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment.

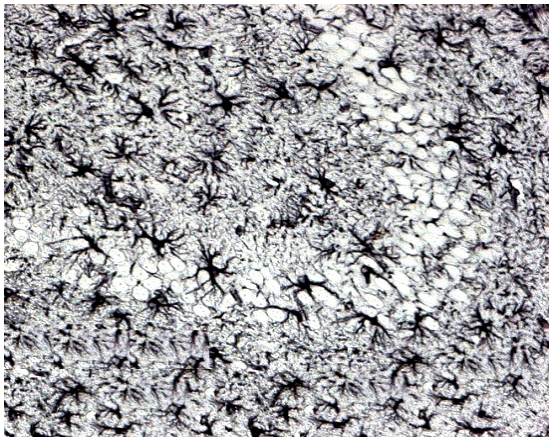


Figure 31: Activated astrocytes invading neuronal cell layers in the CA3 area of the hippocampus of TNF-α Ko mice 5 days post KA

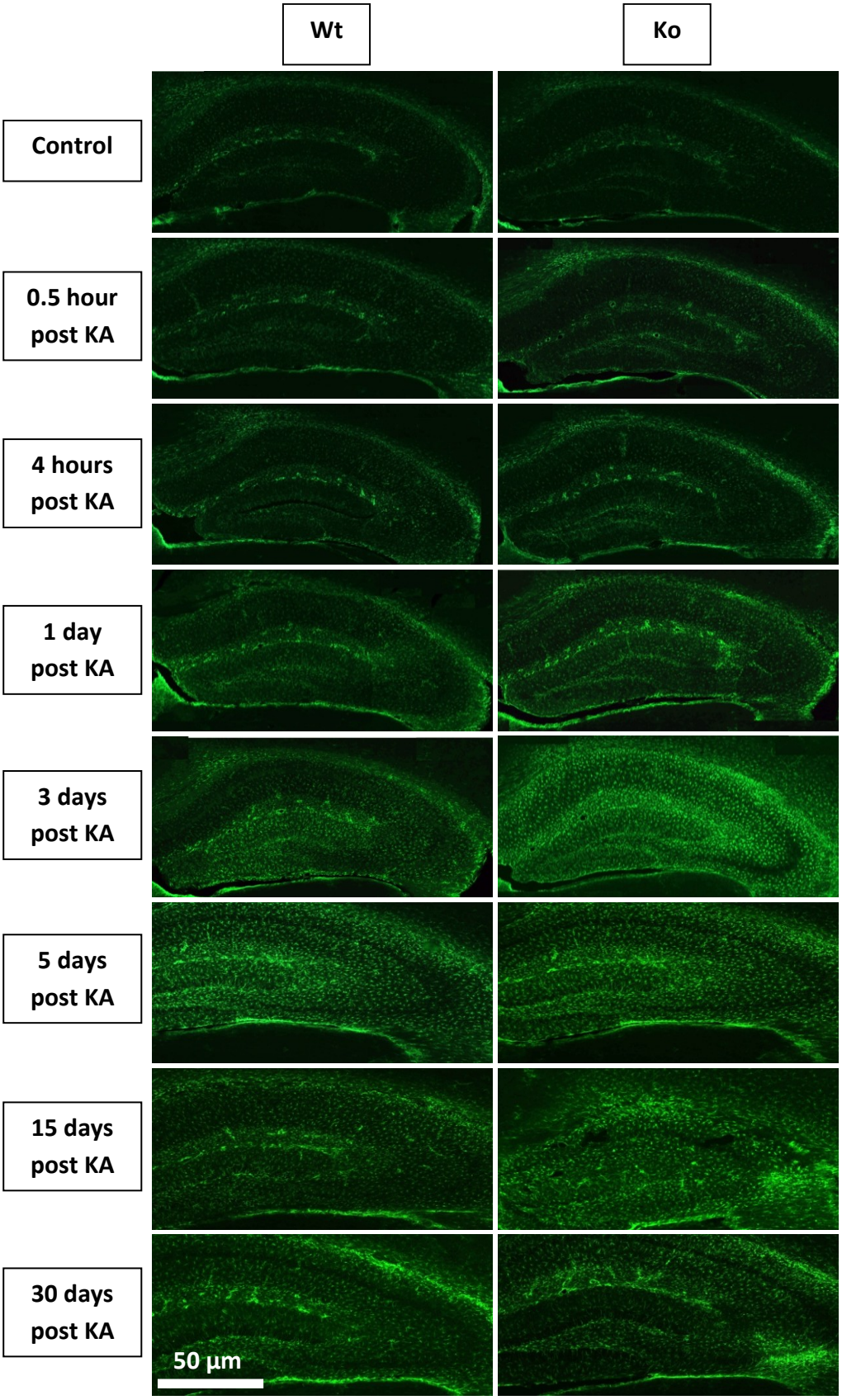


Figure 32: Astrogliosis (GFAP positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment (Magnification 5x)

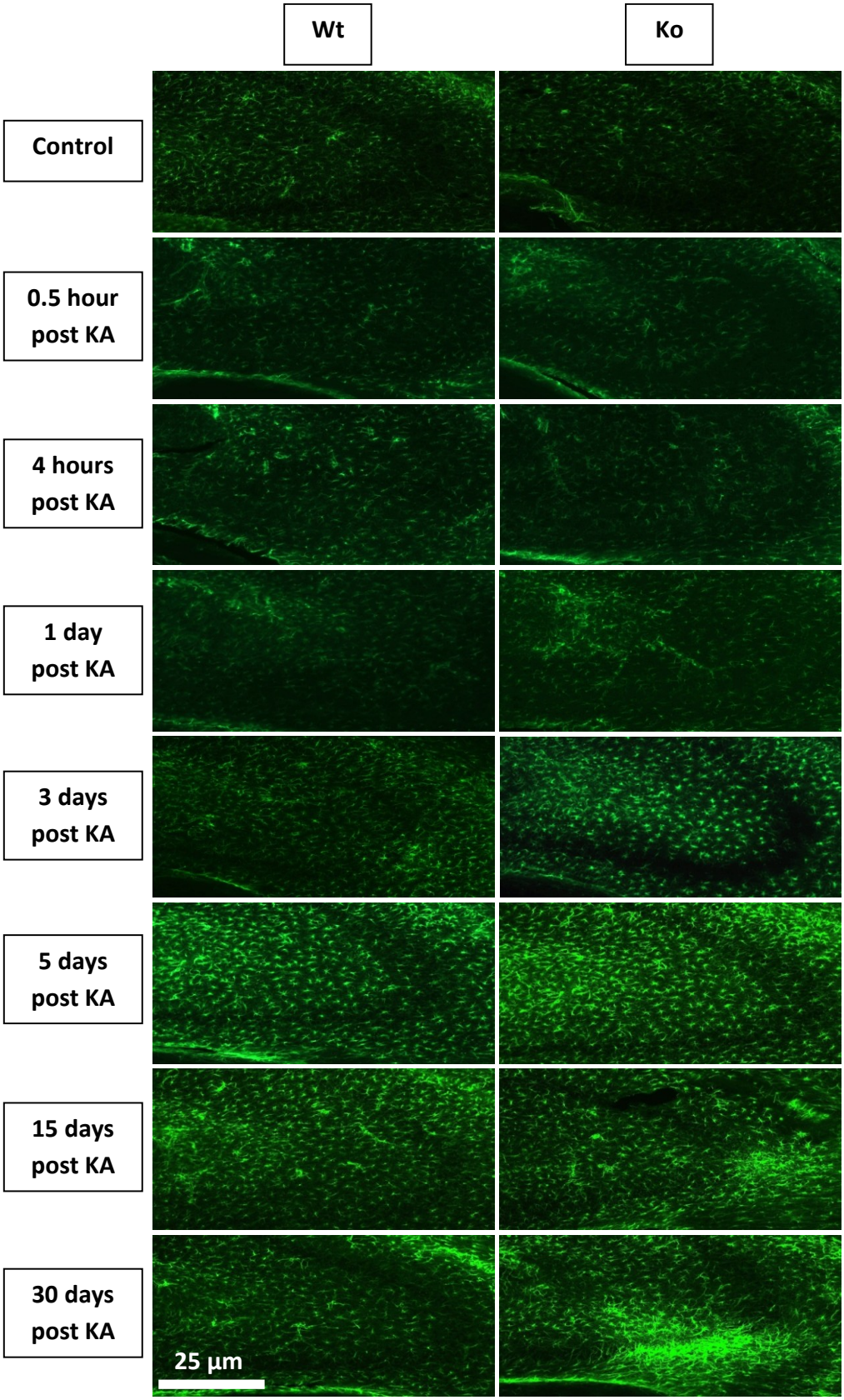


Figure 33: Astrogliosis (GFAP positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment (Magnification, 10x)

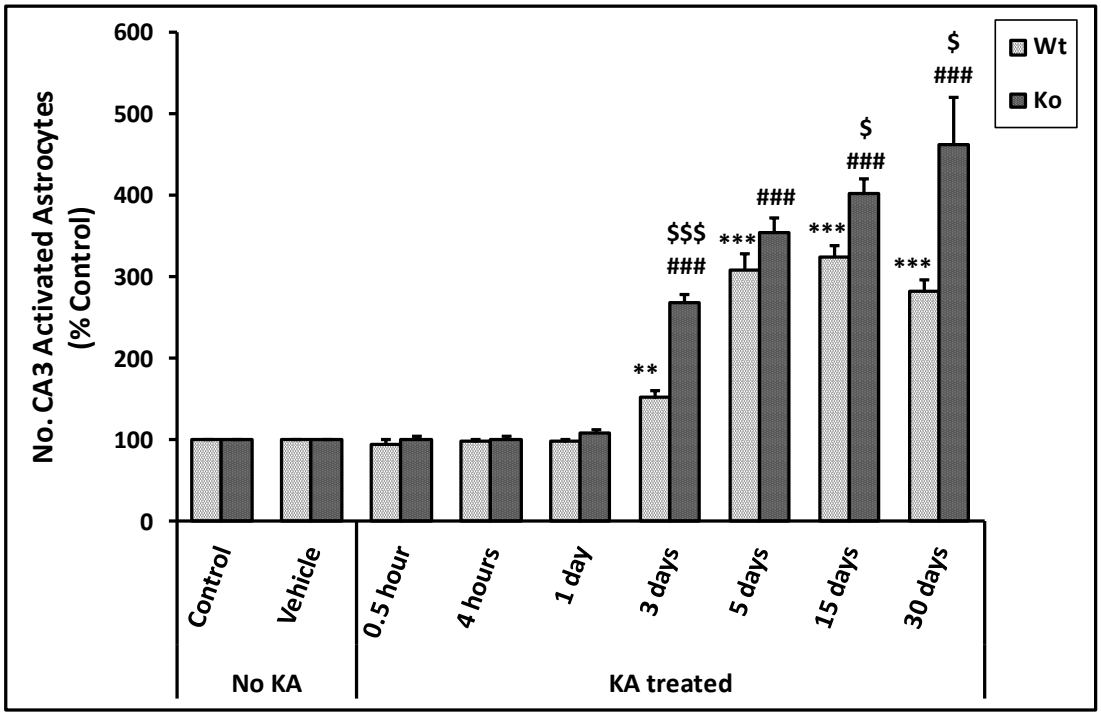


Figure 34: Semi-quantitative counting of astrocytes in the CA3 area (GFAP positive cells) in hippocampi of Wt and TNF-α Ko mice before and after KA treatment

Data represent the Mean ± SEM, (n = 7-9), Vehicle = Distilled Water
**** Significant from Wt Control at P<0.01, *** Significant from Wt Control at P<0.001, ### Significant from Ko Control at P<0.001, $ Significant from respective Wt KA treated at P<0.05, $$ Significant from respective Wt KA treated at P<0.01.**

3.7 Hippocampal Levels of NFκB and AKT

To better understand the mechanism behind the increased neurodegeneration in TNF-α Ko mice, a short term study was conducted and we employed Western blotting to examine NFκB and AKT expression at 1 and 5 days post KA treatment. Both NFκB and AKT expression was detectable by Western blotting in hippocampal supernatants among all groups of mice with or without KA treatment. There was no difference in AKT expression among all groups of mice. Five days after KA treatment, TNF-α Ko mice showed significantly higher NFκB expression than Wt mice (Figure 35).

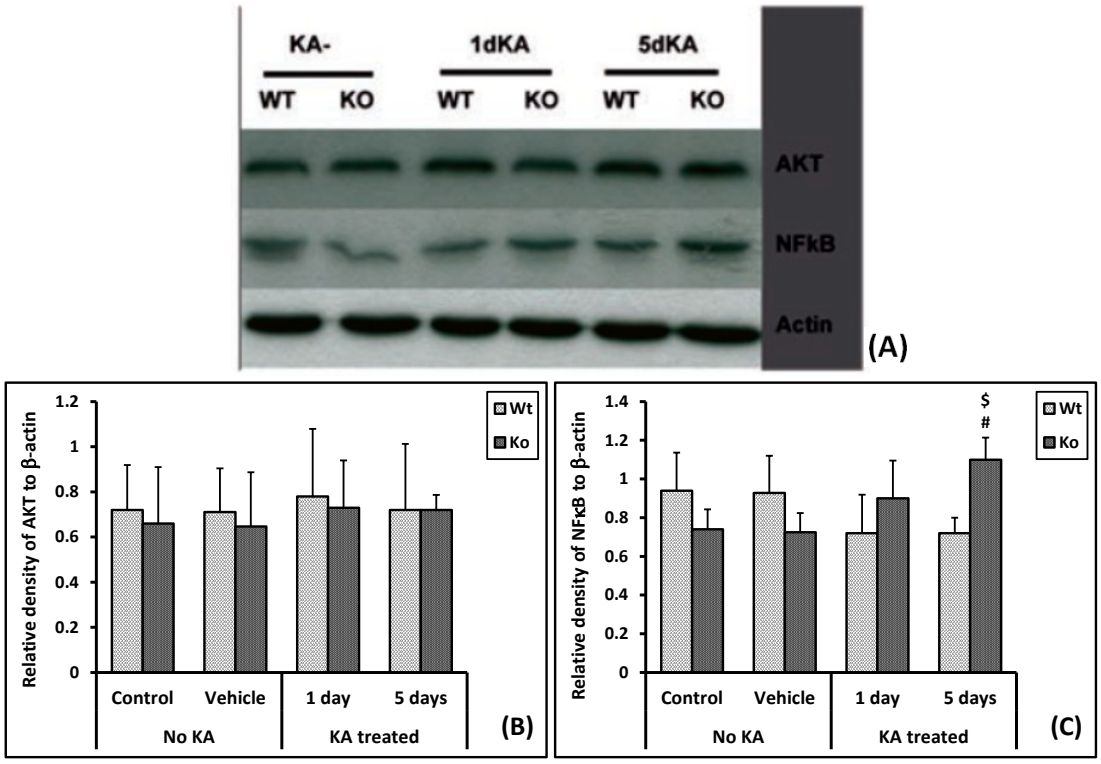


Figure 35: NFκB and AKT expression in hippocampi after KA treatment detected by Western blotting.

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
 # Significant from Ko Control at P<0.05, $ Significant from respective Wt KA treated at P<0.05.

To further confirm our finding from Western Blotting, we also measured the NFκB production at 1 and 5 days post KA treatment by ELISA. The results confirmed that there was a significant increase in KA-induced NFκB expression in hippocampi of TNF-α Ko mice five days after KA treatment. There was no difference between the two groups of mice one day after KA treatment (Figure 36).

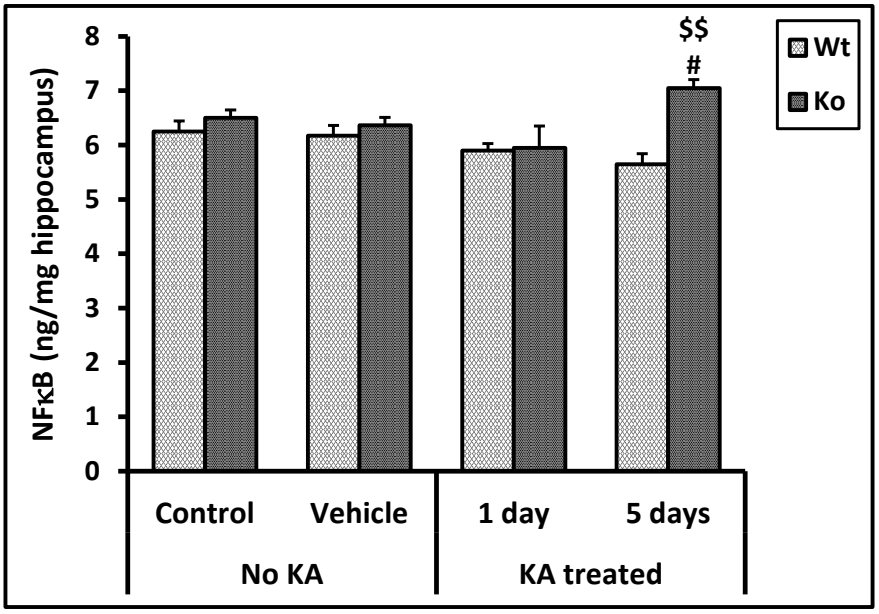


Figure 36: NFκB production in hippocampi after KA treatment measured by ELISA.

Data represent the Mean ± SEM, (n = 9-14), Vehicle = Distilled Water
 # Significant from Ko Control at P<0.05, $$ Significant from respective Wt KA treated at P<0.01.

Chapter 4: Discussion

Neurodegeneration is a common feature of a large number of diseases that come under the umbrella of "neurodegenerative diseases"; and involve loss of nerve structure and function. The prevalence of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease is increasing, but exact mechanisms and effective treatments are lacking. The burden of these neurodegenerative diseases is growing, as the population ages, with huge economic and human costs (Atkinson, 2010).

The causes of neurodegenerative diseases are mostly unknown, and even when they have been identified, the mechanisms by which the diseases start and progress remain speculative. Multiple components are linked to the pathogenesis of neurodegenerative diseases including protein aggregation (Rubinsztein, 2006), mitochondrial dysfunction and oxidative stress (Lin and Beal, 2006), diet components and neurotoxins (Brown et al., 2005), vascular disorders, excitotoxicity (Salinska et al., 2005), and neuroinflammation (Wyss-Coray and Mucke, 2002).

In general, inflammation is a protective response to various cell and tissue injuries to destroy and remove the detrimental agents and injured tissues, thereby promoting tissue repair. However, when inflammation is uncontrolled, it can cause excessive cell and tissue damage ultimately leading to destruction of normal tissue and chronic inflammation (Fischer and Maier, 2015).

Neuroinflammation was previously considered as a passive response to neuronal damage. However, several studies proved that inflammation may be a basic mechanism driving the progressive nature of multiple neurodegenerative diseases (Olmos and Llado, 2014).

Tumor necrosis factor alpha (TNF-α) was originally identified as a factor that leads to rapid necrosis of transplantable tumors in mice (Carswell et al., 1975) and now it is considered as a key player in the initiation and orchestration of inflammation and immunity. TNF-α is known as a powerful proinflammatory cytokine with stimulatory activities for most cells of the immune system (Wajant et al., 2003). Its role in the central nervous system (CNS) was not observed until 1987, when microglia was found to produce TNF-α (Frei et al., 1987).

In the healthy CNS, TNF-α participates in crucial physiological processes such as injury-mediated microglial and astrocyte activation (Merrill, 1991), regulation of blood brain barrier permeability (Sedgwick et al., 2000), febrile responses (Leon, 2002), glutamatergic transmission (Pickering et al., 2005), synaptic plasticity (Santello and Volterra, 2012), learning and memory (Beste et al., 2010), sleep (Krueger, 2008), food and water intake (Plata-Salaman, 2001), and astrocyte-induced synaptic strengthening (Santello et al., 2011). In pathological conditions, TNF-α expression increased in the brain following acute injury (ischemia, trauma), infection, neurodegeneration, and chemically induced neurotoxicity (Allan and Rothwell, 2001; Viviani et al., 2004). However, other findings support a neuroprotective role for TNF-α. In particular, knock-out mice lacking both TNF receptors or TNFR1 receptors only (Bruce et al., 1996; Gary et al., 1998; Lu et al., 2008) show exacerbated ischemic and excitotoxic brain damage. Thus, a controversy exists between the neurotoxic and the neuroprotective roles of TNF-α in the CNS.

The present study was conducted to elucidate the role of TNF-α in neurodegeneration and to follow up the sequence of events occurring in the

neurodegenerative process. This is achieved by intranasal kainic acid (KA) treatment to induce neurodegeneration in TNF-α Ko mice and C57BL/6 Wt mice and study the progress of the neurodegeneration at different time points (0.5 hr, 4 hr, 1, 3, 5, 15 and 30 days) in order to have a better understanding for the neurodegenerative process in presence and absence of TNF-α. Our results show that TNF-α Ko mice were more susceptible to kainic acid (KA)-induced neurotoxicity, as demonstrated by more severe seizures, changed behavior, more degenerating neurons in hippocampi, as well as more glial activation, severe oxidative stress and NO production with malfunctioning defensive mechanisms. Additionally, KA-treatment up-regulated the expression of NFκB to a greater degree after 5 days in KA treated TNF-α Ko mice when compared with KA treated Wt mice. We concluded that TNF-α deficiency worsens KA-induced neurotoxicity and that TNF-α may play a protecting role in KA-induced neurotoxicity via the regulation of the NFκB signaling pathway.

Neuronal excitation involving the excitatory glutamate receptors is recognized as an important mechanism underlying neurodegeneration (Wang et al., 2005). Excitotoxic cell death is commonly induced experimentally by the administration of kainic acid. KA is a non-degradable analog of glutamate and 30-fold more neurotoxic than glutamate. It is a potent agonist affecting the AMPA/kainate class of glutamate receptors (Chihara et al., 2009; Wang et al., 2005). Excitotoxic injury appears to be mediated predominantly by an excessive influx of calcium into neurons through ionic channels (Mayer et al., 1984). Calcium entry into neurons plays a critical role in seizure genesis (McNamara, 1992; Meyer, 1989).

Administration of KA to rodents resulted in recurrent seizures, behavioral changes and subsequent selective degeneration of neuronal populations in the brain (McKhann et al., 2003; Tripathi et al., 2009). Astrogliosis and microglial activation with subsequent cytokines and other inflammatory molecules production are the other characteristics of KA-induced neurodegeneration.

In the present study, KA treated TNF-α Ko mice displayed rapid onset of seizures and more severe seizures than KA treated Wt mice. This immediate and prolonged response is considered the first indication that TNF-α Ko mice were more sensitive to KA-induced neurotoxicity than Wt mice.

Neuronal injury is the main characteristic of KA-induced excitotoxicity. KA-induced damage seriously impacted the hippocampus, which is particularly vulnerable to KA-induced neurotoxicity due to the high density of its kainate receptors (Darstein et al., 2003). CA3 region has the highest abundance of kainate receptors, the activation of which can elevate the concentration of reactive oxygen species (ROS) and impair the normal function of mitochondria (Carriedo et al., 2000; Lauri et al., 2001; Reynolds and Hastings, 1995). CA3 neurons are directly excited by stimulation of their KA receptors, and indirectly by increased glutamate efflux secondary to KA stimulation of mossy fibers. Moreover, depolarization of CA3 pyramidal cells my lead to endogenous glutamate release in the CA1 region (Ashwood and Wheal, 1986; Kim et al., 2000). CA3 synchronization produces spreading epileptiform activity that extends to CA1 and other limbic structures (Bausch and McNamara, 2004; Ding et al., 1998).

In this study, neuronal degeneration started as early as 1 day post KA treatment in TNF-α Ko mice and continued in a progressive way up to 30 days. KA treated C57BL/6 Wt mice showed degenerating neurons 3 days post KA in CA3 area, while KA treated TNF-α Ko mice showed degenerating neurons in both CA3 and CA1 regions. The neuronal damage was more severe and widely extended in TNF-α Ko mice specifically at later time points (15 and 30 days post KA treatment)

CA1 pyramidal neurons receive two distinct excitatory inputs that are capable of influencing hippocampal output, and thus involved in spatial memory and memory consolidation (Iijima et al., 1996; Speed and Dobrunz, 2009). Damage in CA3/CA1 regions of hippocampus induced by KA mainly results in spatial learning deficits.

In our study mice were exposed to a battery of behavioral tasks before and after KA treatment. The behavioral tests include Elevated Plus Maze (EPM) for anxiety and exploration assessment, Open Field test for locomotion and rearing evaluation and Y-Maze for spontaneous alternation and short term memory assessment.

In the EPM, TNF-α Ko mice showed changed behavior (5, 15 and 30 days post KA treatment) in the form of increased frequency and time spent in open arms, less frequency in closed arms and increased number of feces in 5 min compared with that of KA treated Wt mice especially at 30 days post KA treatment. At all time points, both Wt and TNF-α Ko mice showed decreased head drops in open arms and risk assessment behavior compared with those observed before KA treatment. This observation reflects decreased exploration in both strains following KA treatment.

However, TNF-α Ko mice showed significantly decreased counts of risk assessment behavior 30 days post KA treatment compared to Wt mice, indicating attenuated ability of risk assessment (Komada et al., 2008).

In the open-field test, both Wt and TNF-α Ko mice showed increased locomotion and rearing counts 3, 5, 15, and 30 days post KA treatment compared with those observed before KA treatment. TNF-α Ko mice showed significantly more locomotion activity compared with KA treated Wt mice at 3 days and 30 days. The highest levels recorded for locomotion and rearing counts were 3 days post KA treatment.

In Y-Maze test, both KA treated TNF-α Ko and Wt mice showed more arm entries at 3, 5, 15 and 30 days post KA treatment compared with those observed before KA treatment. Similarly, the highest arm entries count was observed 3 days post KA treatment. This increase in arm entries count is because of the hyperactivity of KA treated mice similar to that observed in the Open Field test. On the other hand, the percent of successful alternation between arms was decreased in both KA treated TNF-α Ko and Wt mice at 3 and 5 days post KA treatment compared to those observed before KA treatment. The deterioration in the successful alternation persisted in TNF-α Ko mice at 15 and 30 days post KA treatment, while Wt mice showed improved levels of alternation at 30 days post KA.

The summary of all behavioral studies is that KA treatment resulted in hyperactivity and reduced risk assessment behavior in both TNF-α Ko and Wt mice. However, this changed behavior was more prominent in TNF-α Ko mice specifically

3 days and 30 days post KA treatment. The short term memory was affected by KA treatment in both TNF-α Ko and Wt mice, but there is some sort of improvement observed in Wt mice, but not in TNF-α Ko mice, specifically at 30 days post KA.

It has been shown that KA treated Wistar rats are impaired in the water maze and object exploration tasks, while hyperactive in the open field test (Gobbo and O'Mara, 2004; Gobbo and O'Mara, 2005). Intra-peritoneal injection of KA into the developing rat brain induced impaired short-term spatial memory in the radial-arm maze, deficient long-term spatial learning and retrieval in the water maze, and a greater degree of anxiety in the elevated plus maze (Sayin et al., 2004).

The deterioration in the cognitive functions of the TNF-α Ko mice may be due to the extensive neuronal loss in the CA3/CA1 areas of the hippocampus. However, the possibility that the absence of TNF-α might have implication in the deterioration of the cognitive functions cannot be ruled out. In the CNS, TNF-α was found to affect AMPA receptor trafficking and hence, may affect synaptic plasticity (Beattie et al., 2002). Several studies demonstrated that synaptic scaling is altered by TNF-α and that changes in long-term potentiation (LTP), neurotransmitter metabolism, and neuro-developmental processes are altered by TNF-α as well (Stellwagen and Malenka, 2006). Our results are in line with a previous study showing that continuous exposure of the brain to TNF-α, even in the adult brain, is essential for maintenance of normal cognitive function (Beattie et al., 2002).

KA-induced excitotoxicity is accompanied by increased activation of microglia and astrocytes. Microglia is the main effector cell type responsible for

immune and inflammatory responses in the CNS. Following the injury, all cells present a metabolic reprogramming to cover the bioenergetic and substrate demand for the trophic/inflammatory processes to take place (Gimeno-Bayon et al., 2014) with the coexistence of various factors. Microglia show a range of phenotypes ranging from the well known proinflammatory activation state to a trophic one involved in cell repair and extracellular matrix remodeling (Domercq et al., 2013). The normal role of microglia could be partly connected to neuroprotection, whereas in pathological conditions microglia may become disease-promoting cells. In KA-induced hippocampal injury, microglial activation is generally believed to contribute to neuroinflammation and neurodegeneration. Astrocytes have functional receptors for the excitatory neurotransmitter, glutamate, and respond to physiological concentrations of this substance with oscillations in intracellular Ca^{2+} concentrations and spatially propagating Ca^{2+} signals. Astrogliosis induced by excitotoxicity has been considered as a marker for neurotoxicity (Batlle et al., 2015).

In the present study, TNF-α deficiency enhanced KA-induced microglial activation in the hippocampus. More activated microglial cells have been shown in KA treated TNF-α Ko mice 0.5 hr to 4 hr post KA treatment compared to KA treated Wt mice. At later time points post KA treatment, the number and intensity of Iba-1 positive cells in the hippocampus increased significantly in TNF-α Ko mice. Three days post KA is the peak point at which microglial activation is observed covering all hippocampal areas. At later time points, activated microglia clustered surrounding and within degenerating neurons, specifically neurons in the CA3 area.

In vitro studies showed that microglial activation was detected following treatment of cultures with pathogenic factors when neither neuronal nor astroglial morphological changes could be observed (Figiel and Dzwonek, 2007). Comparative studies carried out on animals treated with neurotoxic doses of TMT revealed that microglial activation precedes the astrocytic reaction and neuronal death (McCann et al., 1996).

Microglial activation was initially considered as a transient event (Streit et al., 1988), however, in this study we have seen that microglial activation persisted up to 30 days which was the latest time point in our study and it may persist for more than this as well. Reports have shown that microglia can remain chronically activated (Huh et al., 2003; McGeer et al., 2003) in a process termed reactive microgliosis. Reactive microgliosis involves microglial activation that occurs in response to neuronal damage, which is then continues and persists by further microglial activation and neurotoxicity. It was found that chronic microglial activation can continue years after MPTP exposure in humans (Langston et al., 1999) and primates (McGeer et al., 2003) despite the fact that the exposure to MPTP was brief.

It is known that microglial activation stimulated activation and proliferation of astrocytes (Batlle et al., 2015). In the present study, astrogliosis occurred earlier in KA treated TNF-α Ko mice (1-3 days post KA) compared to KA treated Wt mice. Similarly, three days post KA is the peak point at which astrogliosis is observed covering all hippocampal areas in KA treated TNF-α Ko mice. At later time points, the intensities of astrogliosis increased gradually and clusters of astrocytes were

found surrounding and invading the degenerating neurons, specifically neurons in the CA3 area.

Oxidative stress is a common characteristic shared across numerous neurodegenerative diseases (Perluigi et al., 2005). ROS play a dual role in determining cell fate. Low levels of ROS may function as second messengers activating pathways that protect cells against apoptotic stimuli. On the other hand, excess ROS are associated with cell death, and blocking ROS often prevents cell death (Chandra et al., 2000). The brain is especially vulnerable to oxidative stress because of its high consumption of oxygen and low levels of endogenous antioxidants (Margaill et al., 2005; Schreibelt et al., 2007). At the cellular level, ROS and RNS can cause DNA and protein oxidation as well as lipid peroxidation. The latter is particularly relevant in the CNS due to the high amount of polyunsaturated fatty acids. ROS also may initiate cell death processes through affecting various signaling cascades (Morgan et al., 2007).

The production of ROS in microglia is derived from multiple sources, such as peroxidases inside the cell, NADPH oxidase on the membrane surface, or the oxidative processes of mitochondria (Circu and Aw, 2009). However, NADPH oxidase is the predominant source of microglial extracellular ROS production (Gao et al., 2002; Qin et al., 2004). It is a membrane-bound enzyme that is dormant in resting phagocytes and can be activated upon exposure to specific stimuli, such as environmental factors (LPS, rotenone, paraquat), endogenous protein toxins (β-amyloid peptide, α-synuclein), and neuronal injury (Babior, 2000). NADPH oxidase

is upregulated in neurodegenerative diseases such as PD (Wu et al., 2003) and Alzheimer's disease (Block, 2008).

Interestingly, microglial ROS production is consistent upon microglial activation, while the production of other factors such as NO, TNF-α or Prostaglandin E2 from microglia is much less consistent across a diverse list of toxins (Block et al., 2004; Gao et al., 2002; Qin et al., 2004). This indicates that microglial-derived ROS may be an essential and common factor of microglial activation.

The activation of KA receptors produces membrane depolarization and results in alteration in intracellular calcium concentrations, which is required to trigger the neuronal death cascade (Brorson et al., 1994). KA administration increased the generation of ROS and RNS. KA stimulated the release of lactate dehydrogenase, an indicator for loss of cell membrane integrity, suggesting that KA induced damage to mitochondrial function. KA also induced mitochondrial loss of glutathione homeostasis and decreased Mn-SOD protein expression. There is growing evidence that free radical generation plays a key role in the neuronal damage (Jones et al., 2002).

The present study showed that KA treatment resulted in a significant oxidative stress at all time points in both TNF-α Ko and Wt mice. This oxidative stress was represented by increased levels of lipid peroxidation products such as MDA, increased NO production, stimulation of antioxidant defensive mechanisms (CAT and SOD), and reactive oxygen species dependent glutathione (GSH) depletion.

Both KA treated TNF-α Ko and Wt mice showed elevated hippocampal MDA levels at all time points compared to their respective untreated controls. However, the oxidative stress in KA treated TNF-α Ko mice started earlier and was significantly higher compared to the respective KA treated Wt mice. Our results showed that ROS production is the very early event occurring as early as 0.5 hr post KA treatment with a peak at 1 day post KA. A previous study showed that the peak of ROS/RNS production occurs 4 hrs after stimulation of the dopaminergic cell culture with LPS (Gao et al., 2002; Harms et al., 2012).

Nitric oxide (NO) is involved in many physiological and pathological processes within the CNS. It can be formed enzymatically from L-arginine by inducible NO synthase (iNOS) in microglia and to some extent also in astrocytes (Wiesinger, 2001). iNOS is scarcely expressed in the brain but it is induced during gliosis in pathological situations including AD (Aliev et al., 2009) and PD (Dawson and Dawson, 1998). ROS, specifically superoxide, can interact with NO to produce peroxynitrite, which has been shown to be very reactive and toxic to neuronal cells (Szabo et al., 2007). Inflammatory mediators, including LPS and some cytokines (TNF-α, IL-1β, and IFN-γ) induce the transcriptional activation of the iNOS gene in astrocytes and microglia via activation of the transcription factors STAT1 and NF-κB (Grzybicki et al., 1996; Hewett and Hewett, 2012; Possel et al., 2000). These factors translocate to the nucleus and bind to response elements present in the iNOS coding sequence. Elevated production of NO by increased activity of iNOS is thought to participate in KA-induced neurotoxicity (Urrutia et al., 2014).

In the present study, we observed that KA is capable of increasing hippocampal NO production in TNF-α Ko and Wt mice. However, TNF-α Ko mice showed earlier (at 0.5 and 4 hr post KA) and significantly higher NO production at 1, 3 and 5 days post KA treatment compared to their respective KA treated Wt mice.

The NO donor, sodium nitroprausside, was found to cause significant neuronal death and demyelination when infused into the brains of mice (Blais and Rivest, 2004). The neurodegeneration caused by sodium nitroprausside is accompanied by microglial activation and the induction of the proinflammatory cytokines such as TNF-α and IL-1β. Animals deficient in TNF-α showed dramatic exacerbation of the sodium nitroprausside induced damage to neurons. The absence of TNF-α resulted in an exaggerated response by microglia. These results proved that early endogenous TNF-α release after the sodium nitroprausside insult is neuroprotective, and demonstrated that microglia of the brain can still be activated in the absence of this important proinflammatory cytokine (Turrin and Rivest, 2006).

Our results are in agreement with previous studies highlighting the neuroprotective effect of TNF-α. One study showed that mice lacking TNF receptors exhibited increased oxidative stress and striatal lesion size following 3-nitropropionic acid (3-NP) administration (Bruce-Keller et al., 1999). Other studies on hippocampal and cortical cell cultures enriched in neurons have shown that TNF-α can protect neurons against oxidative stress and the cytotoxic effects of glutamate and β-amyloid (Furukawa and Mattson, 1998; Mattson, 1997).

As our results showed enhanced oxidative stress following KA treatment, we aimed to check the status of the endogenous antioxidant defensive mechanisms. Glutathione, catalase and superoxide dismutase are the three most important defense mechanisms because the body can produce more of them as needed when certain free radicals are present. Glutathione is the most abundant endogenous antioxidant.

Our results showed that the basal levels of hippocampal GSH were significantly higher in TNF-α Ko mice compared Wt mice. After KA treatment and as a response to the developed oxidative stress, Wt mice showed significantly elevated hippocampal GSH levels as a defensive mechanism. This response was transient and after 3 days of KA treatment, GSH levels declined and nearly depleted at 5, 15 and 30 days post KA. On the other hand, TNF-α Ko mice did not show the transient elevation of hippocampal GSH levels at all. Hippocampal GSH levels in TNF-α Ko mice were significantly reduced gradually 0.5 hr, 4 hr, 1, 3 and 5 days post KA. It was further reduced and nearly depleted 15 and 30 days post KA. These results suggested that GSH defensive mechanism is malfunctioning in TNF-α Ko mice compared to Wt mice.

Similar to GSH, the basal activity of hippocampal CAT were significantly higher in TNF-α Ko mice compared to Wt mice. CAT activity, as a defensive mechanism, was significantly low at 0.5 and 4 hr and was activated at 1 day post KA in TNF-α KO mice in response to their higher oxidative stress and persisted for up to 5 days post KA. Hippocampal CAT activity was then significantly reduced again 30 days post KA compared to their untreated controls and respective KA treated Wt mice. These results showed attempts from the TNF-α Ko mice to antagonize the

emerging oxidative stress. However, these attempts worked well in the early stages but failed to persist more than 5 days post KA treatment.

Higher hippocampal SOD activity were observed in KA treated Wt mice 4 hr and 1 day post KA treatment. This early response was not observed in TNF-α Ko mice that showed significantly higher hippocampal SOD activity only at 3 days and 30 days post KA treatment compared to their untreated controls. These time points represent the critical points in the process of KA-induced neurotoxicity for TNF-α Ko mice. At three days post KA treatment, severe gliosis and significant neuronal death became evident and at 30 days post KA treatment severe neuronal loss was observed in TNF-α Ko mice compared to their respective KA treated Wt mice. At these critical time points the TNF-α Ko mice tried to antagonize the increasing oxidative stress without any success.

It has been documented that prior exposure to a sub-lethal dose of TNF-α makes cells resistant to a subsequent TNF-α challenge. The mechanism by which this TNF-α mediated cellular resistance occurs is related to the enhancement of the intracellular antioxidant capacity (Zimmerman et al., 1989). Examples of this are increased activity of mitochondrial manganese superoxide dismutase (Wong and Goeddel, 1988), and other protective proteins, such as Bcl-2-related family member A1 (Karsan et al., 1996). The molecular basis of the TNF-α induced cellular tolerance is not fully understood at present. One possible mechanism may involve TNF-α induced generation of ROS by leakage from the mitochondrial electron transport chain (Schulze-Osthoff et al., 1992). These intracellular ROS could promote rapid induction of intracellular GSH synthesis (Shi et al., 1994). An

interesting study showed that brief exposure to TNF-α caused a transient depletion of GSH. However, prolonged exposure to TNF-α was found to increase GSH levels (Rahman et al., 1999; Rahman et al., 1996). Elevation of GSH levels has also been observed in cultured rat hepatocytes following treatment with TNF-α, which protected the cells against the cytotoxic effects of further oxidant stresses (Imanishi et al., 1997).

Several studies highlighted the neuroprotective effects of TNF-α in different experimental animal models of neuronal injury such as ischemic tolerance (Nawashiro et al., 1997; Zimmermann et al., 2001) and glucose deprivation-induced injury, and these effects seem to involve attenuation of the elevation of intracellular Ca^{2+} (Cheng et al., 1994). Other potential mechanisms of TNF-α mediated neuroprotection include stimulation of antioxidant pathways (Wilde et al., 2000). Induction of the mitochondrial antioxidant enzyme Mn-SOD by TNF-α has been reported in a wide variety of cell types and has been assumed to function as a central protective mechanism against TNF-α induced apoptosis (Barger et al., 1995; Cheng et al., 1994; Mattson et al., 1997).

Based on the previous findings, it is clear that deficiency of TNF-α renders the body more susceptible to oxidative stress. May be as a compensation, the basal levels of GSH and CAT were higher in TNF-α Ko mice compared to Wt mice. Although GSH levels were higher initially in TNF-α Ko mice, lack of TNF-α makes GSH a malfunctioning antioxidant in these mice. The exact mechanism by which TNF-α regulates GSH still need to be studied.

Some peripheral studies showed an inverse relation between TNF-α and Catalase. For example in a study on cardiomyocyte hypertrophy, it was found that TNF-α as a hypertrophic stimulus can downregulate catalase (Murtaza et al., 2008). In another study, the hepatic catalase expression was also significantly decreased in after TNF-α treatment (Beier et al., 1992). Indeed, in livers of rats treated for 5 days with TNF, catalase activity was reported to be significantly decreased (Yasmineh et al., 1991). In agreement with the latter reports, our finding of elevated hippocampal catalase levels in TNF-α Ko mice could be, in addition to increased oxidative stress, due to the absence of TNF-α in these mice.

Neurodegeneration is associated with increased inflammatory mediators in the CNS such as in AD, MS… etc (Bennett and Stuve, 2009; Rojo et al., 2008). A large number of inflammatory mediators including NO, TNF-α, IL-6, IL-1β, IL-12, IL-18, TGF-β and IL-10 are released by activated microglia and astrocytes after KA treatment (Zheng et al., 2011). Altered expression of cytokines in response to brain injury has diverse actions that can exacerbate, mediate, reduce or inhibit neuronal damage and influence the disease development (Colton and Wilcock, 2010; Kato and Walz, 2000; Kerschensteiner et al., 2009). Results from studies using KA model also indicated that cytokines are involved in neuron-glia intercommunication and manipulation of pro- and anti-inflammatory cytokines can modify the outcome with regard to the seizure activity, behavioral changes as well as the neuropathological consequences (Chen et al., 2004; De Sarro et al., 2004; Swanson, 2009).

IL-1β is a proinflammatory cytokine produced in abundance by activated microglia. Accumulating evidence in experimental neurodegenerative models

indicates that IL-1β is pivotal in the excitotoxicity leading to neuronal death (Liu and Hong, 2003). Primary cultured microglia are significantly activated by KA with increased IL-1β levels and IL-1β can mediate KA-induced excitotoxic injuries to hippocampal neurons in vitro and in vivo (Zheng et al., 2009). On the other hand, IL-6 was found to bear both pro- and anti-inflammatory functions. It appeared to be a critical factor in early phases of CNS insults, taking part in the orchestration of attempts for tissue repair (Amor et al., 2010). IL-6 mRNA was increased in the hippocampus, cortex, amygdale, and meninges, and IL-6 receptor was upregulated in the hippocampus in the rat brain after KA-induced status epilepticus (Lehtimaki et al., 2003).

In the present study, only KA treated Wt mice showed initial elevation of hippocampal TNF-α levels starting from 0.5 hr till 5 days post KA treatment. Both KA treated TNF-α Ko and Wt mice exhibited an initial increase in the levels of hippocampal IL-6 and IL-1β at 0.5 and 4 hr post KA treatment compared to their respective untreated controls. During this period, most of the mice exhibited seizures. Later on, the hippocampal IL-6and IL-1β levels declined.

IL-12 was found to be produced by activated microglia in response to KA, and IL-12 deficiency may alleviate hippocampal injury upon KA challenge, indicating a critical role of IL-12 in excitotoxin-induced brain injury (Chen et al., 2004). Moreover, expression IL-12 was increased in IL-18 deficient mice as compared with controls in association with increased microglial activation, suggesting that the null role of IL-18 in excitotoxic injury may be compensated by the upregulated levels of IL-12 (Zhang et al., 2007). In the present study, the levels

of hippocampal IL-12 was elevated 0.5 and 4 hr post KA treatment in KA treated TNF-α Ko and Wt mice compared to the respective untreated controls. This increase is followed by a sharp decrease that happened earlier in TNF-α Ko mice (1 day post KA) compared to Wt mice (3 days post KA). At 5, 15 and 30 days post KA; the levels of IL-12 in Wt mice were comparable or less than the normal levels. While the IL-12 levels in TNF-α Ko mice returned to their normal levels at 3 and 5 days post KA treatment, then became significantly lower than their respective untreated controls and KA treated Wt mice.

IL-10 is an anti-inflammatory cytokine that suppresses proinflammatory cytokines production such as IL-1β and TNF-α and iNOS, in cell cultures (Aloisi, 1999; Ledeboer et al., 2000). It also represses expression of MHC and co-stimulatory molecules, and inhibits their antigen presenting functions (Sabat, 2010). The main function of IL-10 in inflammatory and autoimmune conditions is the limitation and termination of inflammatory response and regulation of differentiation and proliferation of immune cells (Bashyam, 2007; Park et al., 2007). IL-10 is therefore considered to be an important anti-inflammatory modulator of glial activation, functioning to maintain a balance between pro- and anti-inflammatory cytokine levels in the CNS (Sawada et al., 1999).

The results of the present study showed that the basal levels of hippocampal IL-10 were significantly lower in control and water treated TNF-α Ko mice compared to control and water treated Wt mice. This observation may indicate that TNF-α Ko mice are less protected against neuroinflammation than Wt mice. Half an hour post KA treatment, TNF-α Ko mice showed a transient elevation in the levels of

hippocampal IL-10, then returned back to normal levels followed by a significant decrease at 30 days post KA treatment compared to their untreated controls and KA treated Wt mice. KA treated Wt mice showed decreasing levels of hippocampal IL-10 starting from 4 hr post KA to 1, 3, 5, 15 and 30 days compared to their respective untreated controls.

The overall results of hippocampal cytokine levels indicate an early stage of neuroinflammation occurring immediately within 0.5 – 4 hr post KA treatment. This neuroinflammation is characterized by elevation of proinflammatory cytokines (IL-6, IL-1β and IL-12) and reduction of anti-inflammatory cytokines (IL-10). These findings are in line with the fact that activated microglia, the main producer of these cytokines, were observed in the hippocampus as early as 0.5 hr post KA treatment. We found that neuroinflammation is occurring before neuronal cell death and this finding add more evidence that neuroinflammation is not a passive response to neuronal injury but it can actively participate in the process of neurodegeneration. The alterations in the levels of cytokines starting from 1 day post KA in both TNF-α Ko and Wt mice may represent changes in the phenotype of microglial activation between M1 and M2 microglia.

We observed that both TNF-α Ko and Wt mice exhibit more or less similar pattern of elevation or reduction of cytokine production. However, in a previous study, they showed significantly lower levels of different cytokines (IL-1β, IL-6, IL-10, IL-12) produced from TNF-null microglia upon stimulation with LPS compared to wild type microglia. They concluded that TNF-α is critical for expression and/or secretion of a number of cytokines and chemokines in brain microglia (Douni et al.,

1995; Harms et al., 2012). The rationale behind these opposite results may be because they were running in vitro model and we are using in vivo model. They also used different source for microglial activation which is LPS and we used KA, and these differences can affect the outcome of the experiment.

Several trophic factors were found to be produced in case of brain injury as a compensatory mechanism. β-NGF is involved in maintaining survival and promoting plasticity of neurons in the CNS (Chen and Swanson, 2003). Our results showed that KA treated Wt mice did not show any changes in the levels of hippocampal β-NGF at all time points, while TNF-α Ko mice showed a remarkable elevation in hippocampal β-NGF levels at 0.5 hr, 4 hr and 1 day post KA treatment after which it returned to its normal levels. This elevation in β-NGF may represent a protective mechanism to prevent the greater neuronal damage observed in TNF-α Ko mice.

It was found that microglia expressed β-NGF and that activation of microglia with LPS further increased the expression of β-NGF (Barouch et al., 2001). Recent evidence indicates that cytokines are potent inducers of β-NGF expression both in peripheral tissues and the CNS and that β-NGF, in addition to its neurotrophic action, also acts as an immune-regulatory agent (Steiner et al., 1991).

Insulin like growth factor-I (IGF-I) is a mediator of growth hormone actions and it has been shown to be an important regulator of cell metabolism, differentiation, and survival. Its neuroprotective potential has been documented in ischemia (Guan et al., 2003), traumatic brain injury (Kazanis et al., 2003), or stroke (Liu et al., 2004), as well as in a number of animal models of neurodegenerative

diseases such as amyotrophic lateral sclerosis (Kaspar et al., 2003), multiple sclerosis (Chesik et al., 2007), Alzheimer's (Torres-Aleman, 2007) and Parkinson's disease (Ebert et al., 2008). Moreover, it is reported that IGF-I is able to ameliorate hippocampal neurodegeneration and protect against cognitive deficits temporal lobe epilepsy (Miltiadous et al., 2011).

Our results showed that the basal levels of hippocampal IGF-I were significantly lower in control and water treated TNF-α Ko mice compared to control and water treated Wt mice. This finding showed that TNF-α Ko mice are less protected against neuronal injury than Wt mice. The hippocampal IGF-I levels of KA treated TNF-α mice were gradually decreased at all time points. On the other hand, KA treated Wt mice showed a transient decrease in hippocampal levels of IGF-I starting within 4 hr post KA treatment followed by returning back to normal levels 1 day post KA. Then the levels were significantly reduced at 3, 5, 15 and 30 days post KA but not as much reduced as in TNF-α Ko mice.

It has been reported that in absence of TNFR1, most of the growth factors were down-regulated. These included VEGF, EGF, IGF-I, and NGF, all of which have been implicated in neuroprotection (Dirnagl et al., 2003). This finding suggested that TNF-α can be pivotal for orchestrating the trophic microenvironment of neuronal cells after injury.

TNF-α has a complex biological role in modulating immune and inflammatory responses. Its functions are mediated through two receptors, TNFR1 and TNFR2. TNF binding with its receptors enables TNFR1-associated death domain

protein (TRADD) to bind to the death domain. Following TRADD binding, NF-κB pathway can be activated. NF-κB is a heterodimeric transcription factor that translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors (Wajant et al., 2003). NF-κB is considered to be a point of convergence in the signaling pathways activated by different survival factors, specially IGF-I (Balaram et al., 1999; Vallee et al., 2003) and TNF-α (Jobin et al., 1999; Mukaida et al., 1990). Activation of NF-κB promotes gene expression that can elicit either neurotoxic or neuroprotective effects. Evidence for this dual role comes from studies that show that TNF-α mediated induction of NF-κB is associated with neuronal survival (Albensi and Mattson, 2000; Barger et al., 1995; Kaltschmidt et al., 1999) or the inability of TNF-α to induce NF-κB is associated with increased neurotoxicity (Botchkina et al., 1999; Sriram et al., 2006). Additionally, the stimulatory effect of TNF-α can be mediated via the AKT signaling pathway. AKT is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration.

In this study, we examined NFκB and AKT expression one and five days after KA treatment. Five days after KA treatment, TNF-α Ko mice showed significantly higher NFκB expression than Wt mice, and there was no difference for AKT expression among all groups of mice.

There is increasing evidence that intracellular ROS can function as second messengers to regulate several downstream signaling molecules, including protein kinase C, MAPK and NFκB (Guyton et al., 1996; Konishi et al., 1997; Schreck et al.,

1991). Under physiological conditions this leads to a temporary activation of signaling pathways. However, abnormally large concentrations of ROS/RNS may lead to permanent changes in signal transduction and gene expression, typical for disease states. NFκB, in particular, is often considered to be a ROS-responsive transcription factor and is recognized as a major player in governing cellular responses to oxidative stress (Janssen-Heininger et al., 2000). Importantly, NFκB is a potent inducer of NADPH oxidase and iNOS and thus contributes to the generation of ROS/RNS under proinflammatory conditions (Morgan and Liu, 2011). It is also well established that NFκB can be activated by NADPH oxidase through ROS intermediates (Brar et al., 2002; Clark and Valente, 2004; Gauss et al., 2007).

Studies using neuron-glia cultures from NADPH oxidase deficient mice have shown that NADPH oxidase initiates an intracellular ROS signaling pathway (Forman and Torres, 2002) that can activate microglia and amplify the production of proinflammatory cytokines, such as TNF-α (Qin et al., 2004). In another study, Min and colleagues (Min et al., 2004) demonstrated that the production of IL-1β, TNF-α and iNOS are attenuated by the addition of the NADPH oxidase inhibitor, diphenylene iodonium. Furthermore, inhibitors of NADPH oxidase are shown to suppress LPS-induced expression of cytokines (IL-1β, IL-6, and TNF-α), iNOS, MAPK, and NFκB phosphorylation (Pawate et al., 2004). Hence, there is a cross talk between NADPH oxidase and NFκB, as both of them can regulate and alter the expression of the other based on the surrounding environment. This interaction between NFκB and NADPH oxidase demonstrates the strong interrelationship between ROS/RNS production and the induction of proinflammatory cytokines,

which results in enhanced cell damage and thus aggravates neurodegeneration (Fischer and Maier, 2015).

A study conducted by Emmanouil and his colleagues showed that there is a difference between the outcome of the induction of neuronal NFκB and glial NFκB, where induction of NFκB in microglia, and to some extent in astrocytes, promotes inflammation and neurodegeneration while induction of neuronal NFκB appears to be neuroprotective (Emmanouil et al., 2009) as shown in Figure 37.

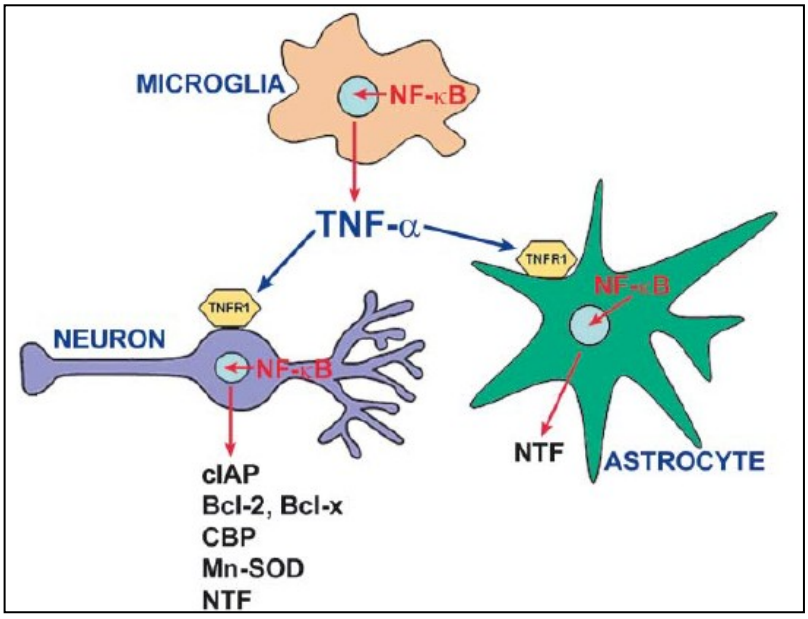


Figure 37: Role of NFκB in neuroprotective actions of TNF-α.

Signals that activate NFκB in microglia (e.g. ischemia, trauma, toxins) induce the production and release of TNF-α. Then TNF-α/TNFR1 interaction on neurons and astrocytes can lead to subsequent activation of NF-κB that promotes neuronal survival by inducing the expression of genes encoding anti-apoptotic proteins (cIAP, Bcl-2, Bcl-x), calcium-binding proteins (CBP), antioxidant enzymes (e.g. Mn-SOD) and neurotrophic factors (NTF) (Figiel, 2008).

In our study, NFκB is upregulated in TNF-α Ko mice 5 days post KA treatment. Of course, this upregulation is not mediated by TNF-α. Based on the previous studies and the results of the current study, the possible mechanism for NFκB upregulation may involve NADPH oxidase and the produced ROS. Moreover, this upregulation of NFκB may be in microglia and astrocytes not in the neurons. This may explain why the balance is going in the direction of inflammation and neurodegeneration. In order to prove these postulations, further studies need to be done. In absence of TNF-α, the neuroprotection mediated by TNF-α / NFκB axis is lost and neurons become more vulnerable for degeneration by the surrounding noxious molecules. We document that NFκB pathway, but not AKT, is important for KA-induced neurotoxicity, which agrees with a previous report (Marchetti et al., 2004) and that TNF-α may exert its neuroprotective role in KA induced neurotoxicity via the regulation of NFκB activation.

Taking together all the results of the present study indicates a sequence of events occurring in the process of KA-induced neurotoxicity within the duration of the study. When KA is administered to the mice, it activates kainate receptors, which are more abundant in the CA3 area of the hippocampus. Activation of KA receptors produces membrane depolarization and results in alteration in intracellular calcium concentrations, which is required to trigger the neuronal death cascade. KA administration increases also the generation of ROS and reactive nitrogen species (RNS) in the neurons. These events act as triggers to activate microglia (M1 phenotype) that respond by the release of inflammatory cytokines, ROS and NO. Activated microglia stimulates proliferation and activation of astrocytes. Excitotoxicity, neuroinflammation and oxidative stress contribute to neuronal cell

death. Dead neurons represent a powerful trigger for further cycles of microglial activation, astrogliosis and neurodegeneration.

TNF-α has been viewed as a neurotoxic agent that is over-expressed in several cases of acute and chronic brain injury. Some studies encouraged therapeutic approaches antagonizing or neutralizing TNF-α effect as a way to avoid its neurotoxic potential. However, with the development of transgenic, knockdown and knockout animals, some studies highlighted the neuroprotective effect of TNF-α by using TNF-α receptors knockout mice. A recent study showed that mice lacking TNFR1 exhibited greater hippocampal neurodegeneration, suggesting that TNFR1 may be protective in KA-induced neurotoxicity (Lu et al., 2008).

In this study, neurotoxicity was induced in TNF-α Ko mice and Wt mice by intranasal administration of KA and the process was followed up at different time points for 30 days. Absence of TNF-α resulted in rapid onset of seizures and severe prolonged seizures. Significant behavioral changes, in the form of higher degree of anxiety behavior, hyperactivity and defects in short-term memory, were observed in TNF-α Ko mice. Severe neurodegeneration in both CA3 and CA1 areas of the hippocampus associated with enhanced and prolonged microglial activation and astrogliosis were shown in TNF-α Ko mice. Neuroinflammation, oxidative stress, lack of antioxidant defensive mechanisms and lack of neuroprotective growth factors were evident in TNF-α Ko mice compared to Wt mice.

From the results of this study we can suggest that: (1) Presence of TNF-α and its early release after an excitotoxic insult is essential in priming microglial cells and

innate immunity to effectively and specifically resolve and phagocytose the initial excitotoxic damage, preventing subsequent secondary damage; (2) Absence of TNF-α impairs microglial activation, which leads to an exaggerated and nonspecific activation of microglia leading to exaggerated production of ROS and NO, accumulation of necrotic debris, amplification of the secondary damage caused by excitotoxin. It was found that the priming of microglial cells upon exposure to neurotoxic insults is TNF-α specific (Blais and Rivest, 2004).

Collectively, the possible mechanisms by which TNF-α may mediate its neuroprotective effects involve (1) regulation of ROS production, (2) activation of astroglia and stimulating neurotrophic factor release (BDNF; Brain derived neurotrophic factor) (Saha et al., 2006), (3) activation of repair processes, (4) stimulation of synaptic currents and thereby mediating neuronal plasticity, (5) activation of NFκ-B pathway, (6) induction of anti-apoptotic factors such as Bcl-2, (7) induction of antioxidant defense pathways (GSH, Mn-SOD), (8) maintainance of calcium homeostasis (Barger et al., 1995; Goodman and Mattson, 1996; Grassi et al., 1994; Mattson et al., 1995; Sriram and O'Callaghan, 2007). TNF-α has been shown to exert direct protective effects on neurons through both TNF receptors. TNFR1 signaling activates and sustains neuronal activity of the anti-apoptotic transcription factor NFκB (Taoufik et al., 2007), and TNFR2 activates an Akt-dependent pathway (Fontaine et al., 2002).

Our results add to the growing knowledge about the multiple factors involved in the neurodegenerative process, specifically excitotoxicity, inflammation and oxidative stress. They also support the growing evidence showing that TNF-α

confers protection for the neurons, specifically for hippocampal neurons which are the focus of our study. Highlighting the neuroprotective side of TNF-α provides an important factor to be considered for future studies on therapeutic approaches involving complete neutralization of TNF-α for treatment of various disorders. We recommend that these treatments should be examined on different areas of the brain to avoid the occurrence of side effects that would arise from complete deactivation of the TNF signal-transduction cascade.

Chapter 5: Final Conclusions

5.1 Summary

Neurodegeneration, which is the loss of nerve structure and/or function, is a complicated process involving multiple components and interconnected mechanisms. Excitotoxicity, neuroinflammation, mitochondrial dysfunction, oxidative stress and protein aggregation seem to be the most common features shared between various neurological diseases involving neurodegeneration (Przedborski et al., 2003).

Inflammation is a complex cascade of self-defensive response to noxious stimuli. Acute inflammation is normally self-limiting and leads to the removal of injurious stimuli and restoration of homeostasis. However, sustained inflammation may result in cellular dysfunction and initiation of disease (Doty et al., 2015).

The resident innate immune cells in the CNS "microglia" provide the first line of defense whenever injury or disease occurs. An acute insult to the CNS (trauma, ischemia, infection, toxic insult) triggers rapid microglial activation with subsequent changes in morphology, gene expression, number and function. Activated microglia produce a spectrum of mediators, such as cytokines, chemokines, ROS, NO and proteases (Prinz et al., 2014).

Of particular importance in neuroinflammatory events, the proinflammatory cytokine, TNF-α, has been demonstrated to act as a key player in the initiation and orchestration of inflammation with broadly ranging activities. TNF-α exerts both homeostatic and pathophysiological roles in the CNS. From its initial description in

peripheral inflammatory responses in 1975, TNF-α is unique in its ability to induce selective necrosis of cancerous cells, while sparing normal counterparts (Carswell et al., 1975). And till now a big controversy exists regarding the role of TNF-α in neurodegeneration. TNF-α overexpression has been implicated in the pathogenesis of several CNS injury including ischemia, trauma, infection, neurodegeneration, and chemically induced neurotoxicity, and potentiates excitotoxic injury to human fetal brain cells. On the other hand, several studies suggest neuroprotective properties for TNF-α based on the findings that absence of TNF-α worsens CNS infections, and knockout mice lacking both TNF receptors or TNFR1 receptors only show exacerbated ischemic and excitotoxic brain damage. Furthermore, dysregulated TNF-α signaling has been implicated in the initiation and/or progression of a number of human diseases (Figiel, 2008; McCoy and Tansey, 2008; Probert, 2015; Sriram and O'Callaghan, 2007). This conflict may be because TNF-α can activate both cell death and survival pathways.

This study was conducted to investigate the role of TNF-α in neurodegeneration and to follow up the sequence of events occurring in the neurodegenerative process. This is achieved by induction of neurodegeneration via excitotoxicity. Excitotoxic cell death is commonly induced experimentally by the administration of kainic acid (KA) which is a non-degradable analog of glutamate and 30-fold more neurotoxic than glutamate. It is a potent agonist affecting the AMPA/kainate class of glutamate receptors. In our model, KA (40 mg/kg body weight) was given intranasally under inhalational anesthesia to TNF-α Ko mice and C57BL/6 Wt mice. The progress of the neurodegeneration at different time points

(0.5 hr, 4 hr, 1, 3, 5, 15 and 30 days) was studied in order to have a better understanding for the neurodegenerative process in presence and absence of TNF-α.

5.2 Conclusions

Intranasal administration of KA to both TNF-α Ko and Wt mice produced the common characteristics of KA induced neurodegeneration in rodents. These include development of recurrent seizures, behavioral changes, selective degeneration of neuronal populations in the brain, oxidative stress, microglial activation and astrogliosis with subsequent cytokines and other inflammatory molecules production (Chen et al., 2002; Zheng et al., 2011).

The most important results and the major differences between TNF-α Ko and Wt mice can be concluded in the following points:

- TNF-α Ko mice were more susceptible to KA induced neurotoxicity as demonstrated by rapid onset of seizures and long lasting severe seizures.
- Both KA treated TNF-α Ko and Wt mice showed significantly changed behavior in the form of altered risk assessment, decreased exploration, hyperactivity and impaired short term memory.
- TNF-α Ko mice changed behavior was significantly prominent specifically 3 days post KA when neuronal loss became evident.
- Wt mice showed some sort of behavioral improvements in performance in EPM and Y-maze at 30 days post KA, while TNF-α Ko mice did not. This may be due to the severe neuronal loss and the continued gliosis observed in TNF-α Ko mice at this time point.

- Hippocampal neurodegeneration was more severe and persisted longer in TNF-α Ko mice.
- Neurodegeneration was observed in both CA3 and CA1 areas in TNF-α Ko mice while it was observed only in CA3 area in Wt mice.
- Absence of TNF-α resulted in exaggerated non-specific activation of microglia.
- Both microglial activation and astrogliosis were intense and extended in TNF-α Ko mice and persisted up to 30 days post KA treatment.
- Neuroinflammation occurred in both TNF-α Ko and Wt mice in the form of increased levels of proinflammatory cytokines (IL-1β, IL-6 and IL-12) and reduced levels of anti-inflammatory cytokines (IL-10).
- Neuroinflammation was observed early in the course of the neurodegenerative process even before neuronal death became detectable.
- KA treated TNF-α Ko mice showed more severe and persistent oxidative stress and increased NO production
- GSH is the main antioxidant defensive mechanism stimulated in response to KA induced oxidative stress in Wt mice. However, this mechanism is malfunctioning in TNF-α Ko mice.
- Absence of TNF-α resulted in increased vulnerability of the cells toward injury by various mechanisms. This may be the reason behind the elevated basal levels of GSH and CAT activity as compensatory mechanisms.
- TNF-α Ko mice showed attempts to overcome the elevated oxidative stress through CAT and SOD.
- All the studied endogenous defensive mechanisms (GSH, CAT and SOD) were observed in the early stages of the neurodegenerative process. However,

continued oxidative stress resulted in failure of most of these mechanisms at late time points especially in TNF-α Ko mice.

- The basal levels of IGF-I in TNF-α Ko mice were lower than Wt mice. This may indicate more vulnerability of TNF-α Ko mice to neuronal injury.
- TNF-α Ko mice showed increased levels of β-NGF following KA treatment, may be as a defensive mechanism to prevent severe damage.
- KA treatment significantly upregulated the expression of NFκB 5 days post KA in TNF-α Ko mice.
- Activation and upregulation of NFκB may be due to the uncontrolled oxidative stress and NADPH oxidase mediated ROS production in TNF-α Ko mice.

In summary, deficiency of TNF-α worsens KA induced neurotoxicity. The neuroprotective effects of TNF-α may be mediated through the regulation of microglial activation, induction of antioxidant defensive mechanisms and regulation of the NFκB signaling pathway.

Chapter 6: Limitations and Future Directions

6.1 Limitations of the Study

There were some limitations in this study including the use of one animal model for induction of neurodegeneration. We preferred to use adult male mice as female and aged C57BL/6 mice were found to be more sensitive to KA-induced neurodegeneration (Zhang et al., 2008), but it will be good if other studies include female subjects as well. Another limitation is the lack of the proper markers to identify M1 and M2 microglia. It is important to appreciate that the use of knockout and transgenic animals helps in understanding the gene function; however there is limited knowledge about the compensatory mechanisms associated with genetically modified animals. Based on the pleotropic effects of TNF-α, there may be some 'unknown' compensatory mechanisms occurring in these mice. So it is better to use animal models with specific inhibition of TNF-α signaling without interference with genetics.

6.2 Recommendations for Future Work

In this study, we were able to demonstrate the neuroprotective effects of TNF-α in KA induced neurotoxicity. Our findings added more to the growing evidence showing that TNF-α confers protection to neurons. However, we cannot generalize these findings to all cases of neurodegeneration and say that the definite role of TNF-α in all neurodegeneration is neuroprotection. Still the precise role of TNF-α in neurodegeneration remains highly controversial due to the complexity and pleiotropic nature of this cytokine and the activities attributed to TNF-α during critical developmental and homeostatic cellular processes. Multiple factors determine

whether TNF-α will exert deleterious or beneficial effects for neuronal survival. These factors may include its concentration, duration of expression (transient or chronic), receptor conformation, tissue type, cellular context and the inflammatory milieu. Because of this multiple variables, caution is necessary when clinically inhibiting the activity of TNF-α, as it is likely that prolonged global suppression of TNF-α and/or TNF-receptor signaling may not be an effective approach to modify the course of neurodegeneration and may even intensify the disease state.

In this study, KA was used to induce hippocampal neurodegeneration, whereas, for future studies, it is important to utilize other models for induction of neurodegeneration to study the neuroprotective role of TNF-α. Furthermore, since we used TNF-α Ko mice as an animal model for deficiency of TNF-α, additional studies involving different ways for TNF-α/TNF-α receptors inhibition, without interference with genetics, will be useful. It is of paramount importance to conduct more research to uncover the possible mechanisms underlying the neuroprotective effects of TNF-α. The NFκB signaling pathway may represent a promising target to be extensively studied at different time points to clarify more the TNF-α neuroprotective effects. Microglial NADPH oxidase and its cross talk with NFκB may represent another promising target to be studied. Future studies focusing on microglial activation phenotypes (M1 and M2) with subsequent morphological changes should be carried out in presence and absence of TNF-α. Finally, further in vivo dissection of TNF-α signaling and its associated receptors in a cell and stage specific context is required for the successful development of selective therapeutic interventions that are safe, specific and efficiently enhance the neuroprotective actions and ameliorate the deleterious actions of TNF-α in neurodegenerative diseases.

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List of Publications

Conference papers:

1. Sara S. Sharkawi and Abdu Adem. (2016). Tumor necrosis factor alpha in kainic acid induced neurodegeneration. International Conference in Educational Neuroscience (ICEN, 2016).

2. Sara S. Sharkawi and Abdu Adem. (2015). Tumor necrosis factor alpha in kainic acid induced neurodegeneration: Cytokines, glia and neurons. (UAEGSRC 2015).

3. Sara S. Sharkawi and Abdu Adem.Impact of Tumor necrosis factor alpha on oxidative stress and growth factors in kainic acid induced neurotoxicity. (UAEGSRC 2016).

Journal papers:

1. Zhang, X. M., Zheng, X. Y., Sharkawi, S. S., Ruan, Y., Amir, N., Azimullah, S., Hasan, M. Y., Zhu, J., Adem, A. (2013). Possible protecting role of TNF-alpha in kainic acid-induced neurotoxicity via down-regulation of NFkappaB signaling pathway. Curr Alzheimer Res, 10(6), 660-669.

2. Sharkawi, S. S. and Adem, A. (2016). Effects of Tumor Necrosis Factor Alpha on Cytokines, Oxidative Stress and Growth Factors in Kainic Acid Induced Neurodegeneration. (Under Review),

3. Sharkawi, S. S. and Adem, A. (2016). Long Term Effects of Tumor Necrosis Factor Alpha on Behavior, Neurons and Glial Cells in Kainic Acid Induced Neurodegeneration. (Under review).